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Contents

	PAGE
C. J. BANKS and H. L. NIXON. Effects of the ant, <i>Lasius niger</i> L., on the feeding and excretion of the bean aphid, <i>Aphis fabae</i> Scop.	703
W. POPLÉ and D. W. EWER. Studies on the myoneural physiology of echinodermata. III. Spontaneous activity of the pharyngeal retractor muscle of <i>Cucumaria</i>	712
R. B. CLARK and J. B. COWEY. Factors controlling the change of shape of certain nemertean and turbellarian worms. (With Plate 12)	731
W. T. W. POTTS. The inorganic and amino acid composition of some lamellibranch muscles	749
G. K. WALLACE. Some experiments on form perception in the nymphs of the desert locust, <i>Schistocerca gregaria</i> Forskål	765
PETER S. B. DIGBY. Flight activity in the blowfly, <i>Calliphora erythrocephala</i> , in relation to wind speed, with special reference to adaptation	776
K. E. MACHIN. Wave propagation along flagella	796
G. M. HUGHES and G. SHELTON. The mechanism of gill ventilation in three freshwater teleosts. (With Plate 13)	807
P. Y. FORTUNE. The effect of temperature changes on the thyroid-pituitary relationship in teleosts. (With Plate 14)	824
J. MAYNARD SMITH. The effects of temperature and of egg-laying on the longevity of <i>Drosophila subobscura</i>	832
LORD ROTHCHILD. Acid production after fertilization of sea-urchin eggs. A re-examination of the lactic acid hypothesis	843
D. W. WOOD. The electrical and mechanical responses of the prothoracic flexor tibialis muscle of the stick insect, <i>Carausius morosus</i> Br.	850
J. E. TREHERNE. The digestion and absorption of tripalmitin in the cockroach, <i>Periplaneta americana</i> L.	862
J. A. RAMSAY. Excretion by the malpighian tubules of the stick insect, <i>Dixippus morosus</i> (orthoptera, phasmidae): amino acids, sugars and urea	871
G. AINSWORTH HARRISON. The adaptability of mice to high environmental temperatures	892
J. SHAW. Further studies on ionic regulation in the muscle fibres of <i>Carcinus maenas</i>	902
J. SHAW. Osmoregulation in the muscle fibres of <i>Carcinus maenas</i>	920
J. B. FREE and YVETTE SPENCER-BOOTH. Observations on the temperature regulation and food consumption of honeybees (<i>Apis mellifera</i>)	930

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EFFECTS OF THE ANT, *LASIUS NIGER* L., ON THE FEEDING AND EXCRETION OF THE BEAN APHID, *APHIS FABAE* SCOP.

BY C. J. BANKS AND H. L. NIXON

Rothamsted Experimental Station, Harpenden

(Received 6 March 1958)

Banks (1958) confirmed that colonies of *Aphis fabae*, attended by the ant *Lasius niger*, multiply more than ant-free colonies on bean plants, in the absence of the aphids' insect enemies. He showed that, when the aphids were ant-attended, they changed their excretion behaviour and that the normal dispersal of adult apterae from young apical growth of bean plants was delayed. No significant differences were found between the reproduction rates of individual ant-attended and ant-free aphids living on leaves of the same age, but the reproduction rates were significantly affected by the age of the plant tissue on which the aphids fed. The increased multiplication of ant-attended colonies was attributed to the delay in dispersal of adult apterae from the young growth, where they reproduced more, to the old growth, where they reproduced less.

Herzig (1937) considered that ant-attended aphids multiplied more than unattended ones because they fed more. He found that *Aphis fabae*, attended by *Lasius fuliginosus* Latreille, and *Aphis sambuci* L., attended by *Lasius niger*, each excreted more honeydew than when unattended, and assumed that the attended aphids therefore absorbed more plant sap. He further assumed that this supposed increase in feeding was the cause of the observed increase in multiplication of ant-attended aphids on a bean plot, and claimed that the stimulation of being fed upon could double or treble aphid numbers.

The experiments described below were made during the summer of 1957 to test the idea that the excretion and feeding rates of *Aphis fabae* on beans are stimulated by the attendant ant *Lasius niger*.

METHODS

Bean plants on which aphids were feeding were made radioactive with ^{32}P so that the aphids took up the isotope and excreted it in their honeydew. The radioactivity of the honeydew taken from them by attendant ants was then compared with that of the honeydew emitted concurrently by unattended control aphids on separate plants.

^{32}P was selected as the most suitable isotope because earlier work had shown that it is relatively simple to grow plants with leaves of the high specific radioactivity required for work of this kind (Watson & Nixon, 1953; Day & Irzykiewicz, 1953). Preliminary experiments showed that ^{32}P is excreted in considerable quantities by *Aphis fabae*

Exp. Biol. 35, 4

living under our experimental conditions. Because ^{32}P is concentrated in the reproductive organs of adult aphids (Watson & Nixon, 1953), losses of radioactivity due to the birth of nymphs would be considerable; to overcome this difficulty nymphs of apterous virginoparae of *A. fabae* were used in all these experiments.

Broad beans (*Vicia faba*) were germinated in wet sand and the seedlings transferred when about 10 cm. high into bottles containing 160 ml. of Hoagland's water-culture solution deficient in phosphorus. Several alate adult aphids were confined to one leaf of each plant in a small plastic box-cage fitted around the whole leaf, and allowed to settle and reproduce for 2 days. The cage was then removed and the alatae and excess nymphs picked off to leave one small group of nymphs on the undersurface of the leaf. ^{32}P , as H_3PO_4 with a small amount of 'carrier' phosphate to facilitate transfer, was introduced into the culture solution at the rate of 300 $\mu\text{c.}/\text{l.}$ of solution. To prevent contamination of the surroundings with radioactive honeydew, a black filter-paper was arranged below the aphids. Black paper was used because preliminary work showed that light reflected from white paper disturbed the aphids, causing them to move about the leaf. The plants were left for 12-48 hr. to allow the leaves and aphids to take up the isotope. When the plants and aphids were ready for use, the black filter-paper below the aphids was replaced with a wire frame supporting a filter-paper stained with bromocresol green, an indicator which changes from yellow to blue when honeydew falls on it (Smith, 1937; Broadbent, 1951), so that the drops could be seen and counted.

Two plants were then placed in a large raised cage standing in a garden and containing bean plants infested with aphids already being attended by ants (*Lasius niger*) which were able to enter and leave the cage through a small hole in the floor. The experiment was begun by trapping ants as they entered the cage and placing one of them on the aphid-bearing leaf of one of the radioactive plants. At the same time the indicator papers were put under both groups of aphids and a stop watch started to time the experiment. The ant was confined to the leaf by a band of grease on the petiole. When it was replete and ready to leave the aphids, it was removed, killed, put aside and immediately replaced by another ant and the whole process repeated. At the end of the experiment the ants, indicator papers, aphids and weighed samples from both leaves were taken for assay. Each sample was dissolved in hot nitric acid, diluted as necessary, and counted in a jacketed G.M. tube attached to a conventional scaling unit.

Ants sometimes investigated with their mouth parts the leaf surface from which aphids had recently been removed. This suggested that the ants might possibly become contaminated with ^{32}P by absorbing some sap directly from the plants. To test this possibility, nine ants were placed on leaves of high radioactivity from which aphids had just been removed. The ants, which were confined three at a time to three leaves in muslin bags, were seen to behave as described at the leaf surfaces. After 1 hr. they were removed, killed and assayed, but gave no detectable increase in count over the natural background rate.

For an average group of about thirty apterous nymphs used in these experiments, the rate of excretion was ascertained by recording the number of drops of honeydew

discharged in 30 min. by 668 nymphs on twenty-four separate plants. On the average, a nymph excreted 0.75 ± 0.05 drop of honeydew in 30 min.; large nymphs, like adult apterae, excreted less often but produced larger drops than small nymphs.

Over periods of 2–3 hr., the excretion rate of a group of apterous nymphs feeding on bean plants in water culture was found to be fairly constant from one $\frac{1}{2}$ hr. to the next, so long as conditions did not vary greatly. Thus, the number of drops of honeydew excreted by eight groups of apterous nymphs (mean, 22 nymphs per group) in successive periods of 30 min. were: 0.51, 0.44, 0.56, 0.47, 0.52, 0.57 (mean, 0.51 ± 0.05 drop per 30 min.).

RESULTS

Series I

The radioactivity of the honeydew collected by a number of ants from a group of aphids (*A*) was compared with that of the honeydew excreted concurrently by a similar group of unattended aphids (*B*) on a separate plant.

Preliminary observations showed a wide variation in the radioactivities of leaves and of aphids feeding on them; many experiments were, therefore, considered necessary to cover the expected range of variation.

In each of twenty-eight experiments, at least three ants (average, four per experiment) were used successively so that the aphids were continuously attended. The duration of the experiments, which varied from 19 min. to 158 min., averaged 58 min. and the average numbers of aphids were 34 ± 3 (ant-attended) and 32 ± 2 (ant-free). The time an ant spent attending depended on the number and size of the aphids of the group; on the average it was 20 min., although in some experiments it was as short as 10 min. Nearly all the ants attended assiduously until their crops were filled.

The radioactivities of the aphids were not correlated with the duration of the experiments but, as expected, were closely dependent on the radioactivities of the leaves on which they fed. In turn, the radioactivities of the honeydew samples were correlated with the radioactivities of the aphids from which they came.

The distributions and means of the radioactivities of the leaf samples, aphids and honeydew samples (expressed in counts per minute) are shown on logarithmic scales in Fig. 1. The distributions for the leaf samples are very similar and their means (*A*, 2.44, *B*, 2.38) are almost identical; those of the aphid radioactivities are also closely similar to each other and the mean radioactivities (*A*, 2.66, *B*, 2.51) also do not differ significantly.

But the distributions of the radioactivities of the two sets of honeydew samples are conspicuously different; that of the ant-free aphids is skew to the left, that of the ant-attended aphids to the right. The two means (*A*, 1.46, *B*, 1.06) are significantly different ($P < 0.01$).

The radioactivity of the honeydew from the ant-attended aphids was apparently double that from the ant-free aphids. It is possible that both groups of aphids over a long period give off the same amount of active honeydew, the ant-attended aphids

producing it quickly to the ants and then becoming exhausted, the ant-free aphids giving off the same amount more slowly. To test this possibility, other experiments were made.

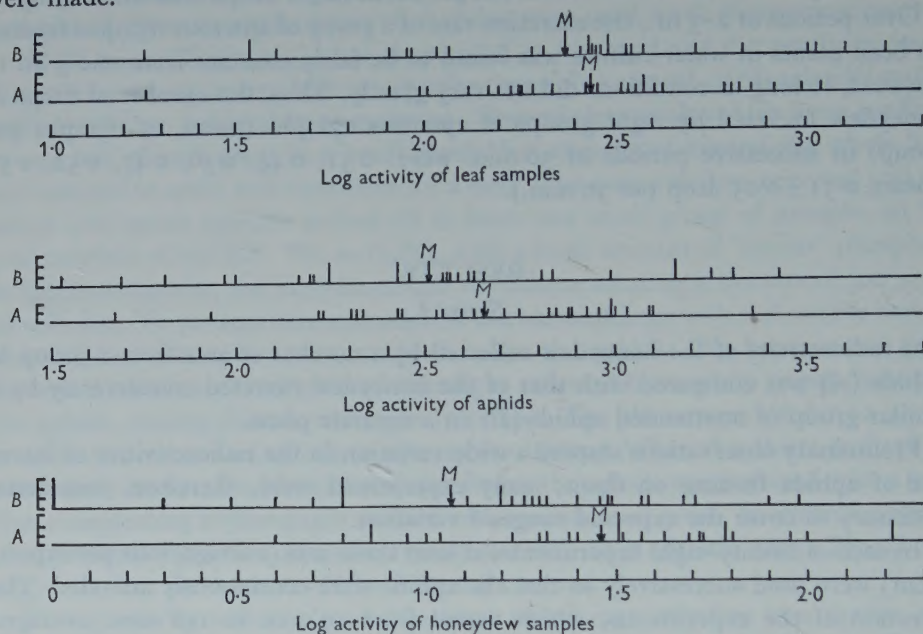


Fig. 1. Logarithmic values of radioactivity of leaf samples, in counts per minute per milligram fresh weight; of aphids, (A) ant attended, (B) ant free, in counts per minute per aphid; and of honeydew samples in counts per minute per drop. (M) mean logarithm. The vertical scales show the number of observations at each value.

Series II and III

In five experiments (Series II), the radioactivity of the honeydew from a group of aphids (A), attended by a single ant, was compared with that from a similar group of ant-free aphids (B); this time, each experiment was divided into five consecutive periods as follows:

*T*₁—a period to establish the 'normal' rate of excretion of the two groups of aphids; it lasted 30 min. and was followed immediately by *T*₂.

*T*₂—the time which a *single* ant spent attending the aphids on the experimental plant A; it lasted 32 min. on the average. When the ant was replete with honeydew and showed signs of leaving the aphids, it was removed and killed.

*T*₃—followed immediately after the removal of the ant. During this period the attended aphids did not emit any honeydew for an average time of 19 min. (range, 15–25 min.). They remained motionless for some time, but then became more and more restless, waving their abdomens and legs in the manner of unattended aphids about to excrete. The ant-free aphids continued to excrete at their normal rate during *T*₃.

*T*₄—began as soon as the first drop of honeydew was excreted by the attended aphids, and lasted for 30 min. During this period, the rate of excretion of the

attended aphids was half the normal rate and the drops of honeydew were conspicuously larger than those produced before the ants attended the aphids.

T_5 —followed immediately on T_4 and also lasted for 30 min. The rate of excretion of the attended aphids tended to return to normal during this period.

Throughout the experiments of Series II, each of which lasted about $2\frac{1}{2}$ hr., the ant-free control aphids excreted at their normal rate. The relative changes in activity of the honeydew from both groups of aphids are shown in Table 1.

During T_1 , both sets of aphids of the Series II experiments produced honeydew whose relative radioactivities were identical; but, as expected from the Series I results, the activity of the honeydew collected by the ants during T_2 was double that from the ant-free aphids.

Table 1. *Relative changes in radioactivity of honeydew from ant-attended (A) and ant-free (B) aphids. Series II. Results of five experiments with 151 (A) and 183 (B) aphids. Series III. Results of six experiments with 177 ant-attended aphids*

Series II

Period	Radioactivity of honeydew per aphid		Mean radio-activity per drop		Rate of excretion per 30 min.		Mean duration (min.)
	A	B	A	B	A	B	
T_1	1.00	1.00	1.00	1.00	1.00	1.00	30
T_2	2.08	1.03	—	1.04	—	1.08	32
T_3	0	1.05	0	1.07	0	0.64	19
T_4	1.06	1.08	1.96	1.11	0.49	1.00	30
T_5	1.69	1.11	2.27	1.15	0.63	1.01	30

Series III

Period	Radioactivity of honeydew per aphid	Mean radio-activity per drop	Rate of excretion per 30 min.	Mean duration (min.)
T_1	1.00	1.00	1.00	30
T_2	2.60	—	—	28
T_3	0	0	0	18
T_4	0.88	0.84	0.50	30
T_5	1.94	2.50	1.00	30

Although the ant-attended aphids excreted nothing during T_3 , the ant-free aphids still excreted honeydew whose radioactivity added to that they had already produced during T_2 equalled that collected by the ants.

During T_4 , each group of aphids excreted honeydew of almost identical radioactivity, but by the end of T_5 , the honeydew radioactivity from the ant-attended aphids was 52% greater than that from the controls.

In interpreting these results, the first problem was to decide whether the attended aphids were exhausted of honeydew by the ants and therefore could excrete only at a reduced rate during T_4 and T_5 ; or whether they replenished the honeydew during T_3 and were holding it back until an ant came to remove it.

The first of these suppositions must be rejected because of the following facts:

(1) the excretion rate of the attended aphids was increasing and tending to return to normal during T_4 and T_5 , as it did in another set of six experiments (Series III; Table 1). (2) During T_3 , the attended aphids were raising and waving their abdomens as if ready to excrete. (3) The drops of honeydew which attended aphids eventually excreted during T_4 and T_5 were conspicuously larger than normal drops. (4) The radioactivity of these drops was double that of normal drops; that is, the rate of excretion is not so significant as the size and activity of each drop. (5) Finally, the experiments of Series I showed that successive ants were able to remove twice as much radioactivity from the aphids they attended as the controls produced freely. It follows, therefore, that the attended aphids had not been exhausted of honeydew for long, but had replenished it rapidly during T_3 ; and that the few which excreted during T_4 and T_5 did so because they could not wait any longer, the others continuing for a while to hold back their honeydew. The experiments show, therefore, that when attended by ants, the aphids produced twice as much radioactivity in their honeydew as ant-free aphids did.

The next problem was to consider the source of the 'extra' radioactivity in the honeydew of the ant-attended aphids; it could come either from the aphids' tissues or from the plant, following an increased uptake of sap by the aphids.

If the radioactivity of the honeydew came from the aphids' tissues, there would be a heavy drain of phosphorus which would have to be replaced by an increased rate of intake and an even more rapid rate of assimilation of phosphorus into the tissues, for we know from the Series I experiments that the radioactivities of the two sets of aphids did not differ significantly at the end of the experiments. There would also be a rapid drain from the tissues of fluid carrying the extra radioactivity and a correspondingly rapid replacement. There is no mechanism in these aphids for the rapid removal of fluid from the gut. The elaborate 'filter chamber' of many Homoptera, whose function is thought to be the by-passing of excess fluid from the anterior to the posterior part of the gut (Wigglesworth, 1953; Waterhouse, 1957), does not occur in *Aphis fabae* and most other aphids, which have not even the simplest of these devices (Weber, 1930); nor have aphids any Malpighian tubes, which are the typical excretory organs of insects (Weber, 1930).

It must be concluded that the ant-attended aphids produced the 'extra' radioactivity in the honeydew by increasing the uptake of radioactive sap from the plants.

It follows, therefore, as Herzig supposed, that the ants directly stimulated both the excretion and feeding rates of the aphids.

DISCUSSION

It was long thought that aphids fed by actively sucking up plant sap, until Kennedy & Mittler (1953) and Mittler (1957) showed that the aphid *Tuberolachnus salignus* (Gmelin) during its normal feeding depends almost entirely on the pressure of sap within the phloem sieve tubes of the plant to force the sap up its stylet food canal.

Mittler concluded that the rate at which the plant forces the sap up the stylet food canal determines the rate of sap uptake and excretion by the aphids. He also

said that the aphids must 'actively swallow' the sap forced up the canal but does not suggest how this could happen. Other workers are under the impression that the aphid has little or no control over its feeding and excretion and that the uptake of sap is continuous. Thus, Waterhouse (1957) supposed that 'the turgor pressure in the plant tissue largely maintains the flow of sap through the stylets into the alimentary canal of the insect so that, once a suitable source of sap is tapped, ingestion is a relatively passive process'; again, Ewart & Metcalf (1956), referring to the article of Kennedy & Mittler, said that 'plant pressure forces large amounts of sap, in the form of honeydew, through the insect's alimentary system'; Bodenheimer & Swirski (1957) also understood that 'the sap is forced into the aphid by its own pressure (Mittler, unpublished data)'.

Mittler (1957) described an experiment in which he anaesthetized feeding *T. salignus* with CO₂ for 24 hr., during which time they did not excrete or become distended. When he severed the inserted stylets of these aphids, however, sap exuded from the cut ends showing that, although it was still under pressure, it had not continued to enter the anaesthetized aphids which had been able to shut off the flow. It has already been stated that *Aphis fabae* can control its rate of excretion by withdrawing the honeydew into the anus when ant-attended (Banks, 1958), and the experiments now described show that the aphids hold back the honeydew until an ant comes to remove it and that they alter their rate of feeding when ant-attended. The excretion and feeding rates are therefore directly controlled by the aphids themselves and are not determined solely by the forces within the plant; the plant merely provides sap under pressure which the aphids are able to tap as required.

The mechanism for controlling sap-intake is probably the so-called 'sucking pump' in the aphid's head (Weber, 1928). This dilatation of the anterior part of the gut lies between the proximal end of the stylet food canal and the oesophagus into which it leads. It is depressed from front to rear and its anterior wall, thinner than the posterior wall, is attached by muscles to the anterior wall of the head. When the muscles contract, they would dilate the lumen of the pump and admit sap under pressure from the stylet food canal; when they relax, the anterior wall would return to its normal position by elasticity and shut off the flow of sap; there are no retractor muscles to close the pump. If the flow of sap into the aphid were continuous, these muscles would be permanently contracted. We suggest that they are normally relaxed so that the pump remains closed; but that periodically they contract to admit sap. Thus, when Mittler anaesthetized aphids, the dilator muscles remained relaxed so that the aphids did not feed and therefore did not become distended.

If this is so sap which has entered the pump would no longer be under pressure and could not, therefore, enter the oesophagus and stomach unless forced. We suggest that the pump opens ventrally to admit sap and then closes; the sap is then forced into the stomach by the closure of the pump, starting at the ventral end. During normal feeding, periods of opening and closing of the pump probably occur at regular intervals, corresponding to the regular emissions of honeydew; when the aphid is ant-attended, the pump would operate more frequently so as to force sap into the stomach more often. But, as Mittler pointed out, the artificial feeding

experiments of Hamilton (1935) and Maltais (1952) showed that aphids can take up limited amounts of fluid which is not under pressure, possibly by suction.

Herzig (1937) supposed that the increased feeding of ant-attended aphids improved their nutrition and consequently increased their reproduction. Waterhouse & Day (1953) quoted Herzig and suggested that the increase in reproduction might be caused by an increased intake of protein. El-Ziady & Kennedy (1956) thought that *Lasius niger* might exercise some control over the physiology of *Aphis fabae* and suggested that the ant 'raises the plane of nutrition of the aphid, perhaps as a result of stimulating its excretion and thereby its feeding as Herzig (1937) thought'.

No differences were found between the reproduction rates of individual ant-attended and ant-free aphids living on leaves of the same age (Banks, 1958). It is concluded that the stimulation of feeding caused directly by attendant ants has little, if any, effect on the aphid's reproduction rate, which is significantly affected, however, by the age of the plant tissue on which it feeds. It seems, therefore, that the reproduction rate of the aphid is affected more by the nature, than by the quantity, of the nutrients which the aphid receives.

SUMMARY

1. To test the idea of Herzig (1937) that the excretion and feeding rates of aphids are stimulated by attendant ants, bean plants (*Vicia faba*), on which groups of nymphs of *Aphis fabae* were feeding, were made radioactive with ^{32}P in water culture, so that the aphids took up the isotope and excreted it in their honeydew. The radioactivity of the honeydew taken from them by attendant *Lasius niger* was then compared with that of the honeydew excreted concurrently by unattended control aphids on separate plants.

2. By increasing their uptake of plant sap the ant-attended aphids produced twice as much radioactivity in their excreta as did the ant-free aphids.

3. The aphids directly control their rates of excretion and feeding, which are not determined solely by forces within the plant.

4. The aphid apparently controls its feeding by the 'sucking pump' in its head. It is suggested that the pump is normally closed but that periodically it opens to admit sap into its lumen and then closes ventrally to force the ingested sap into the stomach. During normal feeding the pump probably opens and closes at regular intervals; but when the aphid is ant-attended it could operate more frequently so as to force sap into the stomach more often. The uptake of sap by normally feeding aphids is apparently not continuous.

We are indebted to Dr K. Mellanby for his advice and to Mr F. G. Smith, Mr R. D. Woods and Mrs C. A. Banks for their invaluable help with the experiments.

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STUDIES ON THE MYONEURAL PHYSIOLOGY OF ECHINODERMATA

III. SPONTANEOUS ACTIVITY OF THE PHARYNGEAL RETRACTOR MUSCLE OF *CUCUMARIA*

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(Received 18 March 1958)

Spontaneous activity is a well attested characteristic of holothurian muscle (Hill, 1926; du Buy, 1936*a, b*; Wells 1942). Some preparations show regular rhythmical contractions and have been used to test pharmacological reagents (Lutz, 1930; Wyman & Lutz, 1930; Iriye & Dille, 1941), while in other preparations the spontaneous activity is so variable that this is not considered possible (Riesser, 1933; Bacq, 1939; Ambache & Sawaya, 1953; Welsh, 1954). For certain preparations there has been no record of spontaneous activity (Morin, 1931; Fischer, 1938; Steinbach, 1940; Galambos, 1941; Prosser, Curtis & Travis, 1951; Prosser, 1954; van Weel, 1955). The variations between different preparations suggest that spontaneous activity is not an inevitable property of holothurian muscle, and in fact Tao (1927) and Buddington (1937) have shown that such activity depends, in part, on the presence of intact nervous tissue. Buddington (1937) studied the cloacal breathing of *Thyone*, and showed that the characteristic patterns of the spontaneous contractions of isolated muscles of the cloacal complex relate to the inherent functions of each muscle in the behaviour of the animal.

The isolated pharyngeal retractor muscle of *Cucumaria sykion* (Lampert) also shows contractions which are considered 'spontaneous' because they occur in the absence of any apparent external stimulation (Pople & Ewer, 1954). These contractions are of the same form and duration as those which pull in the tentacular crown of the entire animal. The experiments reported below show that these contractions are neurogenic in origin and may be reflexly elicited, but that their typical pattern depends upon the presence of a 'centre' within the ganglionic mass which forms the retractor motor complex.

ANATOMY OF THE PREPARATION

The following description is limited to those structures which have a direct influence on the activity of the retractor muscle and is not a complete account of the mechanisms associated with the pharyngeal complex of *Cucumaria*. When

C. sykion is feeding the crown of tentacles is extended, the retractor muscles (Fig. 1a, r) are stretched and the pharyngeal portions of the longitudinal muscles of the body wall (l) are contracted. Withdrawal of the tentacles into the animal is brought about by a slow contraction of the retractor muscles which results in a folding in of the pharyngeal mass. A swift, partial withdrawal can also occur; this is brought about by an initial quick contraction of the retractor muscles and may or may not be followed by a slow, complete contraction of the muscle. When withdrawn the

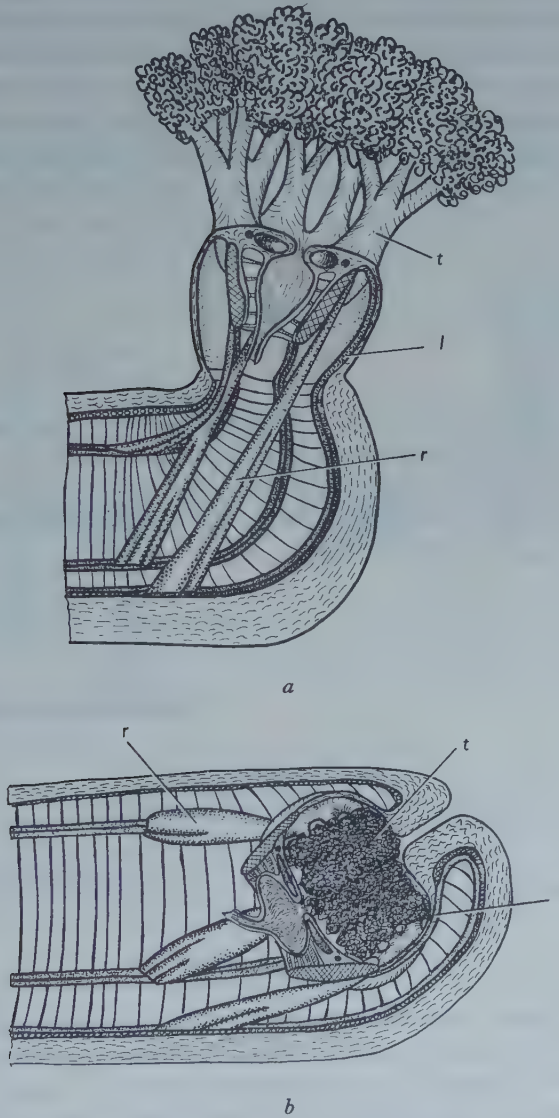


Fig. 1. *C. sykion*. Diagrammatic sections of the pharyngeal region to show relations of retractor muscles (a) with tentacles expanded, and (b) with tentacles retracted. l, Longitudinal muscle; r, retractor muscle; t, tentacle.

tentacles are compressed within the pharyngeal mass (Fig. 1 *b, t*), the retractor muscles have contracted to a shorter length and the pharyngeal portions of the longitudinal muscles are stretched.

The standard preparation used in this investigation consisted of the mid-ventral ossicle with its attached retractor and longitudinal muscles, the two associated tentacles together with the portion of buccal membrane between them and the nervous supply associated with these structures. A diagrammatic representation of the afferent nerves to the retractor motor complex in this preparation is shown in Fig. 2. It has been assumed that severing the retractor nerve in the region 'a-a'' de-afferents the motor complex and that cauterization into the base of the retractor muscle, at the position of the motor complex, denervates the muscle. It has not proved possible to separate the radial nerve from the overlying longitudinal muscle, so that every reference to either radial nerve or longitudinal muscle applies to that structure in association with the other.

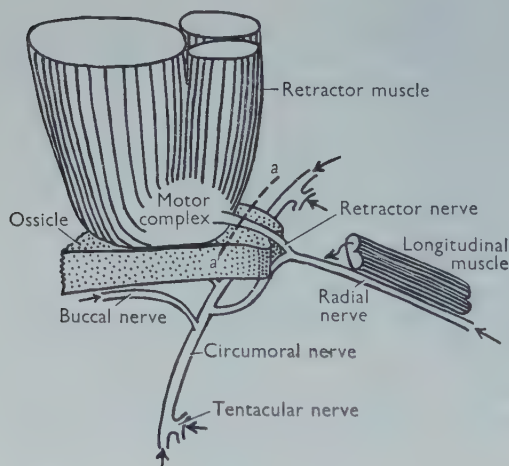


Fig. 2

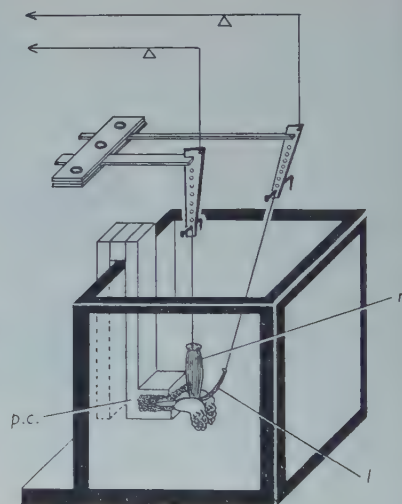


Fig. 3

Fig. 2. *C. sykon*. Diagram of the motor complex at the base of a pharyngeal retractor muscle and its associated nerve supply. Arrows indicate the directions of afferent input to the motor complex. *a-a'*, For explanation see text.

Fig. 3. Diagram of a standard preparation of retractor muscle, *r*, and longitudinal muscle, *l*, set up in a Perspex trough to show the method adopted to apply constant stretches to the two muscles. *p.c.*, Perspex clamp holding ossicle.

METHODS

The methods used were in general the same as those described in earlier papers in this series (Pople & Ewer, 1954, 1955), but modifications were made in the technique of holding the preparation. The most successful way was to grip the ossicle in a piece of foam plastic which was then squeezed into a slot in a vertical Perspex clamp (Fig. 3). This ensured that there was no pressure on either the tentacles or buccal membrane. To be able to stretch either the longitudinal or the retractor

muscle to a controlled length, terylene threads from the free ends of the muscles were tied to pins. Each pin was then slipped through one of a series of holes in a strip of celluloid fixed to a flat spring. By this means different stretches could be applied to either muscle. The contractions of the muscles, which bend the springs, were recorded in the usual manner.

The work of Millott (1954) has emphasized that echinoderm nerves are sensitive to direct stimulation by light. As will presently be seen, this is true for retractor-muscle preparations of *Cucumaria*. For this reason all experiments were conducted in a light-tight dark room; a heavily shaded neon light was used for short periods when it was necessary to check the progress of an experiment. When needed, a general low-intensity light stimulus was provided by a 40 W. fluorescent strip-lamp having an intensity at the preparation of 4-6 lumens/sq.m. A number of animals were dissected under a dim light known to be below the threshold intensity of light stimulation for the preparation; this was, however, discontinued because it was found that, after the first 2 hr., the behaviour of such preparations was no different from those dissected under normal illumination.

SPONTANEITY AND THE CHARACTERISTICS OF THE SLOW CONTRACTION

The most common 'spontaneous' contraction, which has occurred in more than 300 preparations used in this investigation, is a slow, complete shortening of the whole retractor muscle. These contractions may be considered 'spontaneous' because they can occur in the apparent absence of any immediate external stimulation. However, the expression 'spontaneous' has become associated with a number of different causal mechanisms: thus there may be 'internal causation' as in a pace-maker mechanism, 'unspecific causation' where there is general, low-intensity, afferent stimulation and 'unknown causation' with the implication that the activity is not explicable in terms of known reflex mechanisms. To avoid any of these implications the term 'slow contraction' rather than 'spontaneous contraction' will be used as far as possible.

The typical slow retractor contraction has two main characteristics which distinguish it from the quick (Q) and the delayed (D) responses previously described for this muscle (Pople & Ewer, 1954, 1955). First, the rate of development of tension (0.05 ± 0.03 g./sec.) is at least ten times slower than that of the Q and D responses, the contraction time at 20-25° C. being 50-120 sec. in contrast to 2-13 sec. for the Q and D responses. The tension developed by the slow contraction is maintained for 2-4 min. and is followed by an even slower relaxation; the whole event (Fig. 4) takes about 10 min. as compared with 1 min. for a Q and D response. Secondly, in a typical slow contraction, there is always a complete shortening and a maximal tension development, whereas the magnitudes of the Q and D responses are determined by the intensity of the stimulus which evokes them. This complete shortening is shown by the muscle when it is subjected to a stretch of a few mm. and to a tension of a fraction of a gram as much as when it is under the maximal stretch and tension that the muscle can withstand without damage.

At the commencement of a slow contraction the muscle shortens at a gradually increasing rate which gives a smooth sigmoid form to the resulting curve. This characteristic clearly distinguishes both the slow and D responses from the Q response which always commences contraction at its maximal rate and so is recorded as an abrupt step. In cases where the slow contraction is released by a specific stimulus it is possible to assess the latent period of the response. Owing to the gradual development of the contraction very exact measurement is not possible, but, by recording at high speed, latency values varying from 8 to 100 sec. have been found. The latent period of the D response is 3-4 sec. and is thus both considerably shorter and more constant than that of the slow contraction.

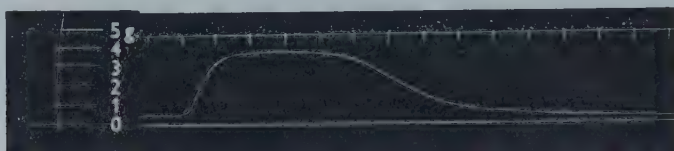


Fig. 4. *C. sykon*. A typical slow contraction of the pharyngeal retractor muscle. Temperature 23° C.; time marker, 1 min.

As well as being distinguished from the Q and D responses by its form, the slow contraction differs also in the way in which it is released. The Q and D responses are only obtained from the retractor muscle when the radial nerve is abruptly stimulated, either electrically or mechanically. Slow contractions can result from a number of other methods of stimulation which are described below, but they also arise in preparations which, left in the dark at a constant temperature and in conditions which exclude mechanical stimulation, are apparently unstimulated.

ORIGIN OF THE SLOW CONTRACTIONS

In order to locate the mechanism which controls the slow contraction, two types of preparations were used.

(a) *The denervated retractor muscle*. If the motor complex of one muscle of a five-muscle preparation is destroyed by cautery, that muscle will produce no further slow contractions even though the remaining four muscles continue to make slow contractions for 48 hr. Cautery of the complex does not destroy the muscle fibres for they will still respond to direct electrical stimulation. Again, if a preparation is treated with 10^{-6} curare (D-tubocurarine chloride, Burroughs Wellcome) the slow contractions cease, but they return after the curare has been washed out of the muscle. The curarized muscle will respond to direct electrical stimulation.

These results suggest that the slow contractions are neurogenic rather than myogenic in origin.

(b) *The de-afferented retractor muscle*. If the retractor nerve of a muscle showing rhythmical slow contractions is severed, the muscle ceases its activity. The ability to produce a slow contraction has not, however, been destroyed completely, for, if

the preparation is left for several hours, it may produce occasional slow contractions. It is possible also to release a slow contraction from such a preparation by a number of unspecific stimuli; for example, by general low-intensity stimulation by light, by draining and replacing the sea water around the preparation, which presumably produces a mechanical stimulation, and also by altering the ionic composition of the sea water.

From these results we may tentatively conclude that the mechanism producing the slow contraction lies in the motor complex, but that the normal occurrence of the contractions is dependent on afferent stimulation.

INHIBITION OF SLOW CONTRACTIONS

A preparation from which the tentacles and buccal membrane have been carefully removed still possesses a considerable afferent supply to the retractor motor complex from the radial and circumoral nerves. If such a preparation is left in the dark the retractor muscle will produce slow contractions for several hours, provided that the longitudinal muscle is not stretched. If, however, the longitudinal muscle is stretched the slow contractions of the retractor muscle cease. When the stretch on the longitudinal muscle is released, the slow contractions of the retractor return (Fig. 5). That stretching the longitudinal muscle inhibits the retractor slow

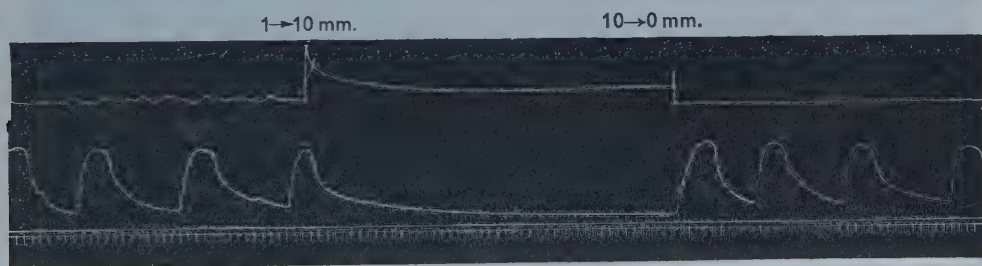


Fig. 5. *C. sykon*. Retractor and longitudinal muscle preparation without tentacles. Upper trace: longitudinal muscle; lower trace: retractor muscle. At the commencement of the excerpt the longitudinal muscle is stretched 1 mm. After 30 min. it is stretched to 10 mm. and after a further 45 min. all stretch on the longitudinal muscle is released. The sudden increase in tension in the retractor muscle as stretch is released is an artifact arising from the handling of the preparation. Time marker, 1 min.

contractions is not unexpected as these two muscles are functional antagonists. But it is also necessary to recognize that the occurrence and form of the slow contractions depend on a balance between excitatory and inhibitory inputs, for, if the longitudinal muscle is held at an intermediate stretch, patterns of partially inhibited slow contractions are obtained. These changed patterns will continue for periods longer than 12 hr. provided that the stretch on the longitudinal muscle is maintained and the changes from one pattern to another are always completely reversible.

The stages in the development of this inhibition are illustrated in Fig. 6. It will be seen that, for a stretch of 1 mm. or less, no change occurs, but that with greater stretches on the longitudinal muscle the pattern of the retractor contractions is altered. These changes are noteworthy. First, the frequency of the contractions is diminished; secondly, the duration of the contractions is prolonged; thirdly, the tension developed may no longer be maximal and finally the tension developed may vary markedly during the course of a contraction. It will be seen that at different stretches there is a close correlation between the duration of the contraction and the duration of the period between contractions. The uneven tension development, shown, for example, in Fig. 6 for a stretch of 3 mm., may perhaps be the expression of a varying balance of inhibitory and excitatory influences.

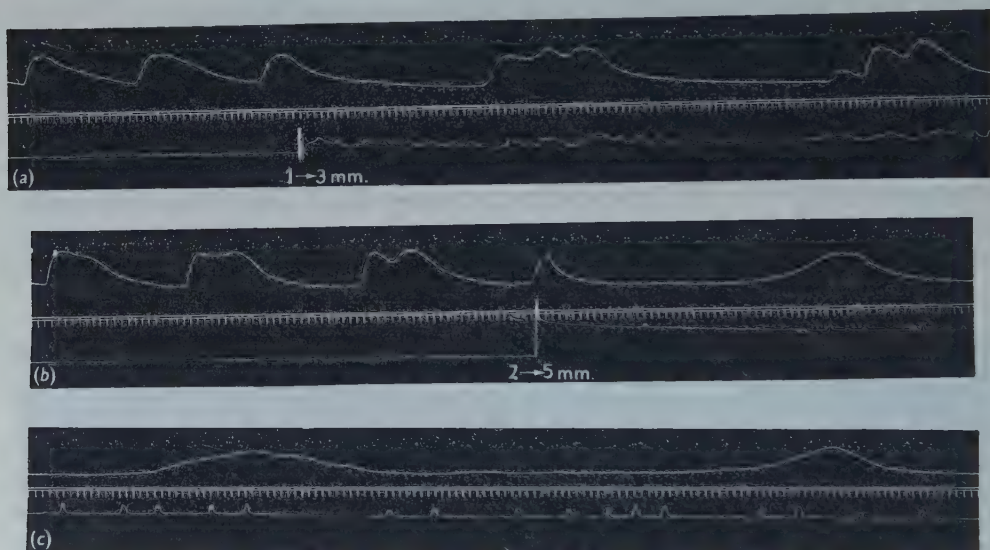


Fig. 6. *C. sykon*. Retractor and longitudinal muscle preparation without tentacles. Upper trace: retractor muscle; lower trace: longitudinal muscle. Time marker 1 min. (a) Stretch on longitudinal muscle increased from 1 to 3 mm.; (b) stretch on longitudinal muscle increased from 2 to 5 mm.; (c) stretch on longitudinal muscle 6 mm. throughout. All extracts are from the same preparation.

Evidence for the total inhibition of an event as uncertain as these slow contractions must inevitably be negative and therefore unsatisfactory. But it is also possible to release slow contractions reflexly by stimulation of the tentacles. The reflex response thus produced contains a further element—a twitch—whose presence is witness to the occurrence of effective stimulation. For this purpose a preparation was used of the mid-ventral pharyngeal structures with the two tentacles attached on either side of the ossicle. Such a preparation shows not only slow contractions but also discrete twitches (Fig. 7a). During these twitches the muscle contracts at the maximal rate found during a slow contraction, but they show neither the initial gradual increase in tension nor the maintenance of tension characteristic of a

slow contraction. Unlike the typical slow contraction, the twitch does not usually involve more than 30 % shortening of the muscle. As the twitches only occur in preparations which have tentacles and buccal membrane intact, they are probably associated with excitation from the sensory tissues which these structures possess. The twitches can occur in the apparent absence of external stimulation, but are more frequent when a preparation is first set up and then become smaller and cease after a few hours. The magnitude of these twitches is variable, unlike the typical slow response.

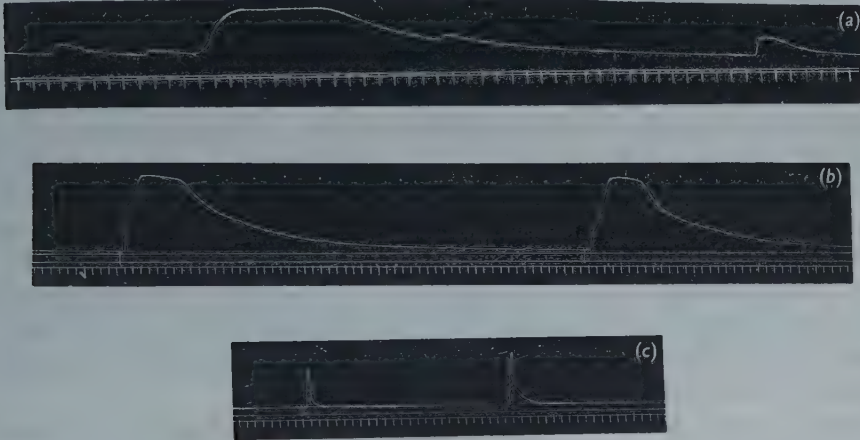


Fig. 7. *C. sykion*. Retractor and longitudinal muscle preparation with tentacles. Responses of the retractor muscle. Time marker, 1 min. (a) Spontaneous twitches and slow contractions with no stretch on the longitudinal muscle; (b) longitudinal muscle not stretched: responses to touching tentacle with a glass rod, first gently and then very gently; (c) longitudinal muscle stretched to 4 mm.: responses to touching tentacle first gently and then with violent rubbing.

If a tentacle is gently touched with a glass rod the retractor muscle will produce a twitch and, provided that the longitudinal muscle is not in a stretched position, this twitch will be followed by a slow contraction (Fig. 7*b*). If the tentacle is violently rubbed a number of times, the size of the twitch may be larger, but there is no difference in the size of the slow contraction produced. If the longitudinal muscle is stretched and the tentacle is touched or rubbed, then the retractor muscle will give a twitch, but it will not be followed by a slow contraction (Fig. 7*c*). Thus it can be seen that, although a gentle touch on the tentacle is sufficient to release a slow contraction, yet if the motor complex be in a state of inhibition due to the stretching of the longitudinal muscle, then even violent stimulation of the tentacles will not produce a slow contraction. It thus seems legitimate to conclude that stretching of the longitudinal muscle produces a real inhibition of 'spontaneous' slow contractions.

SLOW CONTRACTIONS RELEASED BY ELECTRICAL STIMULATION

Preparations can be obtained in which, for long periods, slow contractions do not occur 'spontaneously'. Two of the commonest used were: (a) a single retractor-muscle preparation with as little buccal sensory tissue as possible and with the longitudinal muscle stretched; and (b) a double retractor-muscle preparation pinned down with moderate pressure upon the tentacles.

In such preparations electrical stimulation of the radial nerve may be followed by a contraction of the retractor muscle which has, in general, the quantitative characteristics of a slow response. In any one preparation both 'spontaneous' and electrically released slow contractions are identical, but there is considerable variation in the quantitative details of a slow contraction from preparation to preparation. This variability is found, moreover, neither with the Q and D responses nor with responses of the muscle to direct stimulation. This suggests that the variability of the slow contraction may be a reflexion of differences in the state of the slow-contraction mechanism within the motor complex; this state will be largely determined by the amount of intact afferent tissue, both excitatory and inhibitory, which inevitably varies from preparation to preparation. This variability and the relatively limited number of stimuli which may be applied to any one preparation makes for difficulties of experiment and interpretation. The results which are reported below have been obtained from a sufficient number of preparations to warrant their acceptance.

A single 2 msec. electrical stimulus applied to the radial nerve can produce a typical slow contraction. The threshold for this slow contraction is always greater than that for both Q and D responses (Fig. 8), and thus it has not been possible by electrical stimulation to release a slow contraction alone. If single stimuli at a fixed voltage, just above threshold for the release of a slow contraction, are given at intervals during the life of a preparation, although the Q and D responses appear regularly, the occurrence or non-occurrence of the slow contraction is to a large extent unpredictable (Fig. 9). However, the longer the time interval since one slow contraction, the greater the chance that a threshold stimulus will release another. This effect is shown in Table 1, which summarizes the results from four preparations tested in this manner. This gradual lowering of threshold for discharge demonstrates a variation in the excitability of the slow-contraction mechanism with time. That this variation is a regular process in any one preparation may be shown by the use of single, regularly spaced test shocks above threshold voltage. In such experiments it is found that the time interval between slow contractions is almost constant in any one preparation, although with different preparations this period may vary from 20 min. to 1 hr.

It was considered possible that regular repetitive stimulation of the radial nerve might produce a more rapid development of the excitability of the centre within the motor complex and result in more frequently elicited slow contractions. To study this question series of stimuli were applied to the radial nerves of different preparations at frequencies varying from $2\frac{1}{2}$ /sec. to 1/10 min. and at various

intensities. After numerous fruitless experiments it became clear that it is not possible to 'prime' the slow-contraction system by way of the pathways in the radial nerve associated with the Q and D responses. Indeed, batteries of stimuli are no more effective than single stimuli in releasing slow contractions.

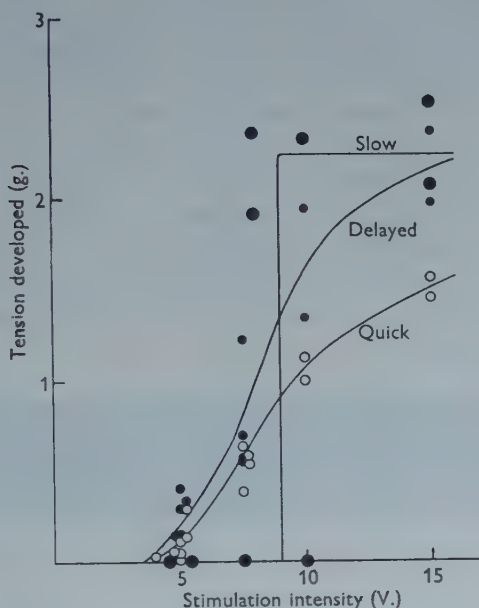


Fig. 8. Graph showing the relationship between the intensity of stimulation and the tension developed by the retractor muscle in response to single stimuli of 2 msec. duration. The Q, D and slow responses are shown separately. Small open circles: quick response; small filled circles: delayed response; large filled circles: slow response.

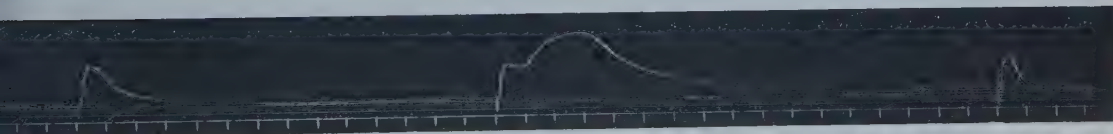


Fig. 9. *C. sykon*. Response of retractor muscle to three single 1 V. stimuli applied to the radial nerve. In all three cases a Q and D response are given, but a slow contraction follows only in the second case. Time marker, 1 min.

Table 1. *Effect of time since a slow contraction on the probability of releasing another by electrical stimulation at threshold*

Time since previous slow contraction (min.)	15-30	30-60	60 and more
Threshold stimuli producing a slow contraction	6	9	9
Threshold stimuli not producing a slow contraction	13	11	3
Percentage effective stimuli	32	45	75

Although the electrically released slow contraction usually has an 'all-or-nothing' character, yet partial or incomplete contractions did occur with rather less than 10% of the stimuli given in experiments studying conditions around threshold. Such partial contractions could not, however, be produced by careful grading of the intensity of the threshold stimuli. They reflect conditions within the motor complex and not the intensity of the releasing stimulus applied to the radial nerve. Similar partial contractions may be obtained if a preparation is stimulated at a voltage above threshold, provided the longitudinal muscle is stretched and, if the stretch be sufficient, the response may be completely inhibited. If, now, the intensity of the stimulus applied to the radial nerve is increased, a slow contraction may again be elicited. This parallels the condition found with 'spontaneous activity' of the retractor muscle, the slow contraction of which could be partially or totally inhibited by stretching the longitudinal muscle, and emphasizes that the activity of the afferent supply to the motor complex is as important in determining the character of the response released by stimulation of the radial nerve as it is for 'spontaneous' slow contractions.

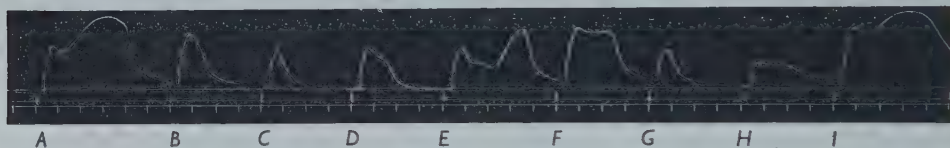


Fig. 10. *C. sykon*. Response of retractor muscle to single 1 V. stimuli applied to the radial nerve at 4 min. intervals. Time marker, 1 min.

Without an apparent change in the background of afferent activity, partial slow contractions may also be elicited electrically in preparations from which typical slow contractions can likewise be obtained. Such contractions are shown in Fig. 10, where it will be seen that after a normal slow contraction at *A*, partial slow contractions were released at *D*, *E* and *F*, to be followed later by further partial contraction at *H* and a normal slow contraction at *I*. These results give the impression that, in certain circumstances, stimuli from the radial nerve may force the slow contraction centre of the motor complex to discharge prematurely.

While it has not been possible to show any consistent influence of stimulation of the radial nerve upon the character of the slow contraction, there are marked effects of the slow contraction upon the relative magnitudes of the Q and D responses. Preparations which show no 'spontaneous' activity and in which the threshold for an electrically elicited slow contraction is very high will produce Q and D responses of constant size to series of stimuli which lie below the threshold for the slow response. Sometimes there is a decrease in the size of the Q response with time, but this appears to be an accommodation effect at the point of stimulation. However, in a preparation giving slow responses, the Q and D responses preceding and following the slow contraction are modified. The commonest modification, seen in preparations making only an occasional slow contraction, is an increase in

size of the D response relative to the Q when the stimulus immediately precedes the slow contraction and the opposite effect 10 min. after the slow contraction. In other preparations, in which there are frequently occurring slow contractions, there

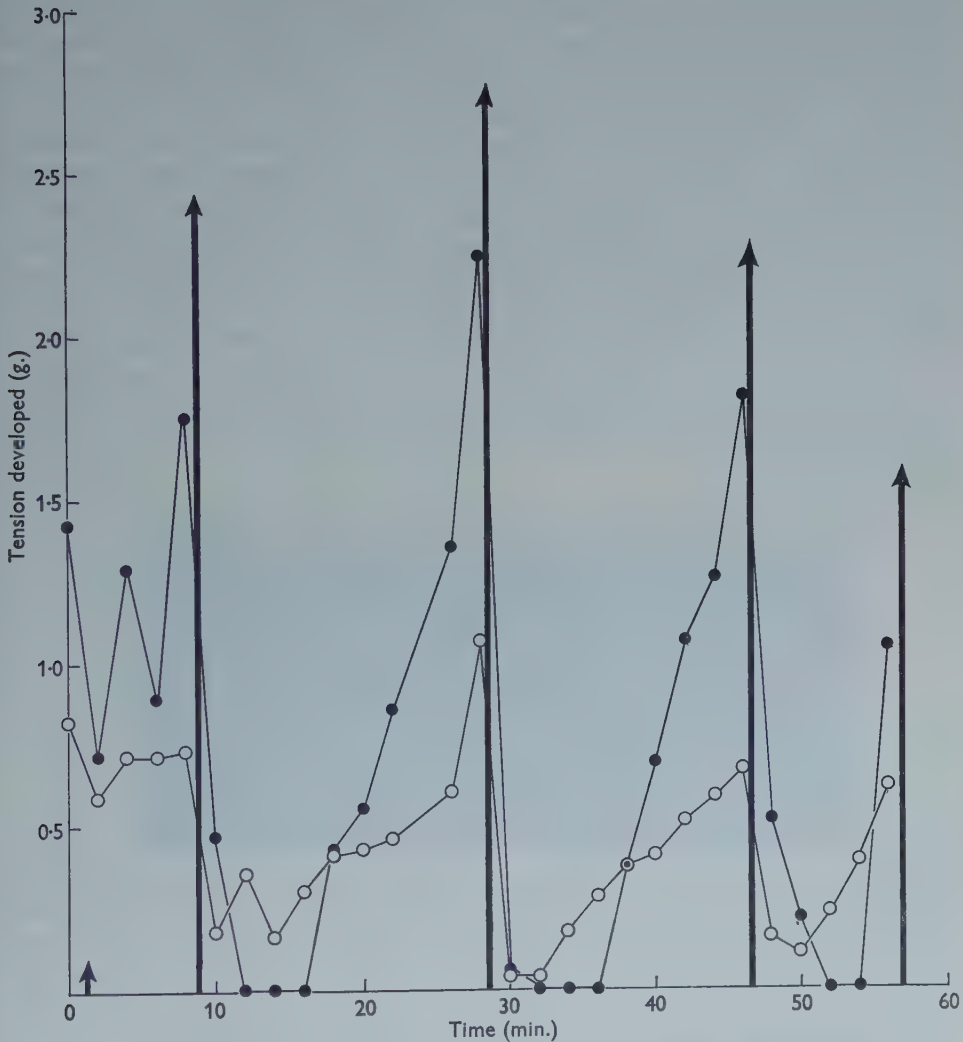


Fig. 11. Graph representing the tensions of the Q and D responses of the retractor muscle to a series of test shocks of 2 V. intensity and 2 msec. duration applied to the radial nerve at 2 min. intervals during a period of spontaneous activity. Open circles: quick response; full circles: delayed response. Arrows show time of onset of slow contractions: their height indicates the maximal tension developed by each contraction.

are increases in the absolute size of the Q and D responses until the slow contraction occurs and then there is a marked decrease following the contraction (Fig. 11). The D response is affected to a greater extent than the Q.

If the radial nerve is stimulated during a slow contraction, only minute Q and D responses will be elicited. This is a mechanical effect as the muscle has developed almost maximal tension due to the slow contraction. It is well known that histologically all fibres of this muscle are parallel, and one would therefore expect that, should the Q and D responses and the slow contraction use different final efferent pathways, simple algebraic summation of tensions would result. As this is not the case, it seems likely that all three responses have indeed common final pathways to the muscle.

The effects recorded with electrical stimulation of the radial nerve support the picture obtained from the earlier results, that the slow contraction mechanism is controlled by a system within the motor complex of the retractor muscle and that in this it differs markedly from the Q and D contractions which are immediate reflex responses arising from stimulation of the radial nerve, albeit the detailed characteristics of these responses may be modified by influences within the ganglionic mass of the motor complex. As the two systems probably have a common final path in the ribbon axons to the muscle fibres they must overlap. This overlap probably lies within the motor complex where intrinsic neurones are known to exist; these neurones have earlier been invoked to explain the characteristics of the D response (Pople & Ewer, 1954).

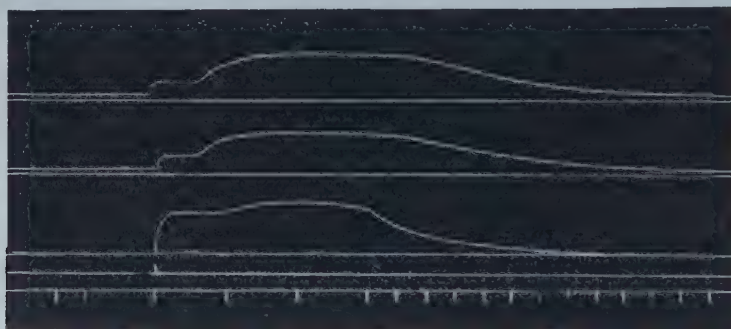


Fig. 12. *C. sykon*. Extract from a preparation of five retractor muscles. Upper trace: right dorsal muscle; middle trace: right ventral muscle; lower trace: mid-ventral muscle. A single 2 msec. stimulus of 8 V. applied to the mid-ventral radial nerve at the time shown by the upper signal marker. Time marker, 1 min.

CONDUCTION AROUND THE CIRCUMORAL NERVE

It has been shown (Pople & Ewer, 1955) that, following stimulation of a radial nerve, impulses are transmitted to the retractor muscles of other radii. Except for a decrease in the tension developed the Q and D responses of a para-radial muscle are similar to those of the muscle whose radial nerve is stimulated. Slow contractions can also be elicited from all the muscles of a five-retractor-muscle preparation by the stimulation of any one radial nerve (Fig. 12). These slow contractions are of the typical 'all-or-nothing' character described above, and do not show the decrement around the ring found in the Q and D responses.

When a radial nerve is stimulated a slow contraction is not always obtained from all the muscles of a five-muscle preparation, although they all show Q and D responses. With subsequent stimulation it will not be the same muscles which fail to give a slow contraction. This failure of a muscle to produce a slow contraction is not due to a dying away of excitation around the ring, for the muscles furthest from the stimulated radius may produce slow contractions while those nearer do not. Thus the release by electrical stimulation of slow contractions around the ring does not depend upon the slow contractions of a neighbouring muscle nor merely on the impulses arriving in the Q and D system.

Table 2. *Times of commencement of slow contractions in different muscles of a five-muscle preparation relative to the occurrence of the 'leader' of each series*

(All times given in minutes. L indicates leading muscle. Arrows indicate possible path of excitation).

Time of occurrence of 'leader contraction'	Interval between successive leader contractions	Pharyngeal retractor muscles				
		Left dorsal	Left ventral	Mid-ventral	Right dorsal	Right ventral
3.0	—	1.2	← L →	1.1	→ 1.1	→ 2.6
14.7	11.7	4.5	L	5.4	3.2	—
31.5	16.8	4.1	L	—	→ 1.8	→ 1.9
47.8	16.3	17.5	← 16.8 ←	L →	10.5	→ 16.4
95.8	48.0	7.5	L	8.6	6.8	6.0
151.3	55.5	→ 0.6 →	0.6	—	2.1	← L
176.7	25.4	1.8	← 0.9 ←	L →	0.5	→ 0.6
206.7	30.0	—	—	3.6	← 1.7	← L
258.8	52.1	→ 1.4 →	1.9	3.1	← 2.3	← L
320.1	61.3	L →	1.6	—	5.0	5.3
346.7	26.6	2.1	← 1.2 ←	L →	1.3	→ 4.4
365.8	19.1	1.8	—	L	23.6	2.7
413.3	47.5	→ 0.6 →	2.9	1.1	← 1.1	← L

This behaviour is paralleled in the activity of an unstimulated five-muscle preparation. When such a preparation is first set up the contractions of the five muscles appear to be independent and at random, but after about 3 hr. the contractions become less frequent and tend to occur in all the muscles together (Fig. 13). In Table 2 the times of commencement of spontaneous slow contractions are shown relative to the moment when the first muscle of a series contracted. It will be seen that usually, although not invariably, the muscles on either side of this 'leader' contract first and are then followed by the distal ones. This is similar to the mode of conduction of the Q and D responses round the circumoral nerve ring (Pople & Ewer, 1955). In ten preparations carefully studied there was no evidence for dominance by any one radius in this spontaneous activity.

A further study of Table 2 shows that the time interval between the occurrence of the slow contraction of the leader and that of either para-radial muscle is long and variable. The shortest interval that has been observed is 12 sec. and the longest several minutes. It seems unlikely that this represents the conduction time of impulses passing round the ring from the leading radius, and must rather be

regarded as a latency in the slow-contraction mechanism of the motor complex of the muscle showing the contraction.

The mode of circumoral propagation implies that some pathway exists which ensures that the discharge of the slow-contraction mechanism of one radius will trigger the discharge of those of other radii, provided their mechanisms are fully excitable. It would be difficult to explain the leader phenomenon in unstimulated preparations without this assumption. Further evidence in favour of this is obtained from a consideration of the size of the Q and D responses accompanying the electrical release of slow contractions in a five-muscle preparation. The Q and D responses associated with slow contractions in radii para-radial to that of the stimulated radial nerve may be smaller than those released by stimuli threshold for a slow contraction, when the para-radial nerves are themselves stimulated. Nevertheless the slow contractions are maximal and do not show the circumoral gradation of the Q and D responses. Moreover, in these conditions, particular retractor muscles may fail to give slow contractions, although the more distal ones do, confirming that the releasing impulses from the leading radius pass independently to all other radii.

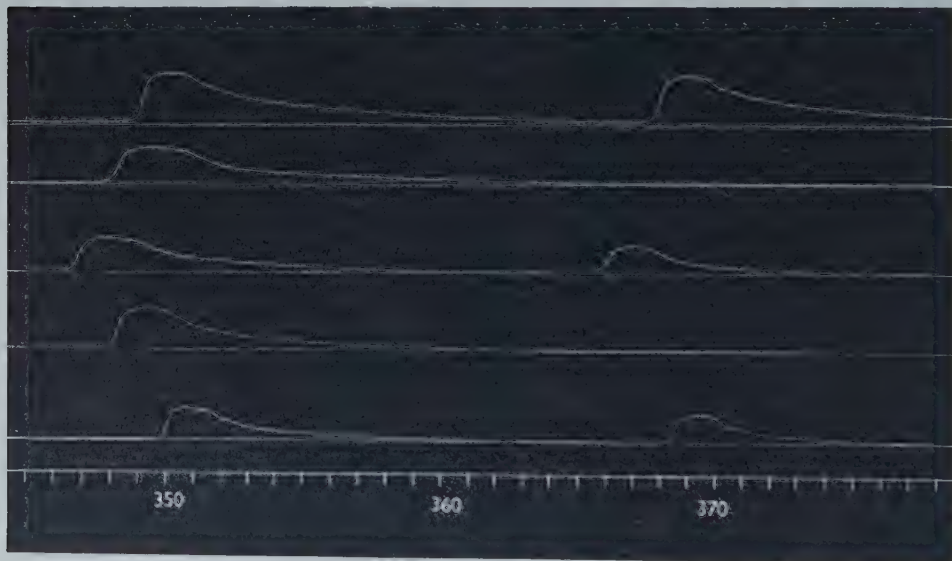


Fig. 13. *C. sykion*. Extract from a preparation of five retractor muscles showing spontaneous activity. Figures indicate time in minutes from setting up preparation. Time marker, 1 min.

DISCUSSION

Batham & Pantin (1954) and Ross (1957) have described the properties of the slow contractions of sea anemones. These show a number of striking similarities to the slow contractions described above, but there are also important differences. In both sea anemones and *Cucumaria* these slow contractions are obtained in response to electrical stimulation and to various unspecific stimuli such as light or touch; both display a long and variable latency; in both cases the slow contractions differ

from quicker contractions which are produced by the same muscle; in both the contractions are smooth and sigmoid in nature and they have very similar time relations: these latter similarities probably reflect the fact that in both cases the slow contractions will work normally against the pressure of a hydrostatic skeleton. But there are many important differences. In sea anemones the thresholds of quick and slow responses to electrical stimulation are the same, in *Cucumaria* they are markedly different; in sea anemones a number of electrical stimuli are required to elicit a slow contraction, in *Cucumaria* a single stimulus may be sufficient; in sea anemones the magnitude of the slow response may be graded by the number of electrical stimuli applied, in *Cucumaria* it is typically all-or-nothing; in sea anemones the maximal tension developed by the slow contraction is only about half that developed by the quick, in *Cucumaria* the slow contraction normally develops maximal tension; in sea anemones the slow contraction may alone be obtained in response to electrical stimulation, in *Cucumaria* it is always accompanied, in these conditions, by Q and D responses.

Batham & Pantin (1954) consider that the slow contraction of a sea anemone has its genesis peripherally, arising from a 'state of excitation in the muscle which, if it reaches a sufficient intensity, is followed after a considerable delay by a smooth contraction'. In *Cucumaria* our evidence suggests that the slow contraction has its genesis within the motor complex of the retractor muscle and not at the muscle or the myo-neural junctions. Moreover, although typically an all-or-nothing response, the slow contraction of *Cucumaria* may be graded, but this gradation appears also to be a function of the co-ordinating activity of the motor complex and not, in any simple manner, determined by the stimulus applied. The slow response is a fragment of the behaviour pattern associated with withdrawal of the tentacular crown, and its partial or complete inhibition by stretching of the pharyngeal portion of a longitudinal muscle may be correlated with the fact that these muscles are stretched when the tentacles are withdrawn and further activity by the retractor muscles is not required.

We have already suggested, in a consideration of the Q and D responses, that the motor complex of the retractor muscle is capable of modifying sensory input in the manner of the central nervous system. The present results emphasize that the motor complex may have an integrative role as well. It is possible at this stage to offer only a formal and hypothetical explanation of how this is effected and of the implications of the phenomena we have observed. It may be assumed that within the motor complex of the retractor muscle there is an arrangement of neurones capable of producing the programme of slow contraction; we may speak of this as the pattern centre (Fig. 14), and visualize it as normally in an excited state. This excitation will only be released by sensory input facilitating its passage through a 'releasing mechanism' or 'gate'. Also it is postulated that there is an inhibitory centre which is excited by the discharge of the pattern centre. The inhibitory centre has, further, certain characteristics such that its excitation only gradually dies away. It also acts upon the gate, but its effects spread beyond this and are responsible for the variations in magnitude of the Q and D responses before and after a slow con-

traction (Fig. 11). Such a system, given a steady input of sensory impulses, would produce rhythmic patterned responses. A marked fall in sensory input would result in occasional, random slow contractions, such as are observed in preparations where the motor complex is isolated. At the same time any sudden increase in sensory input might result in a full, patterned response provided the level of inhibition was low enough. But incomplete responses, reflecting some balance of excitation and inhibition, can be released by electrical excitation (Fig. 10). Further,

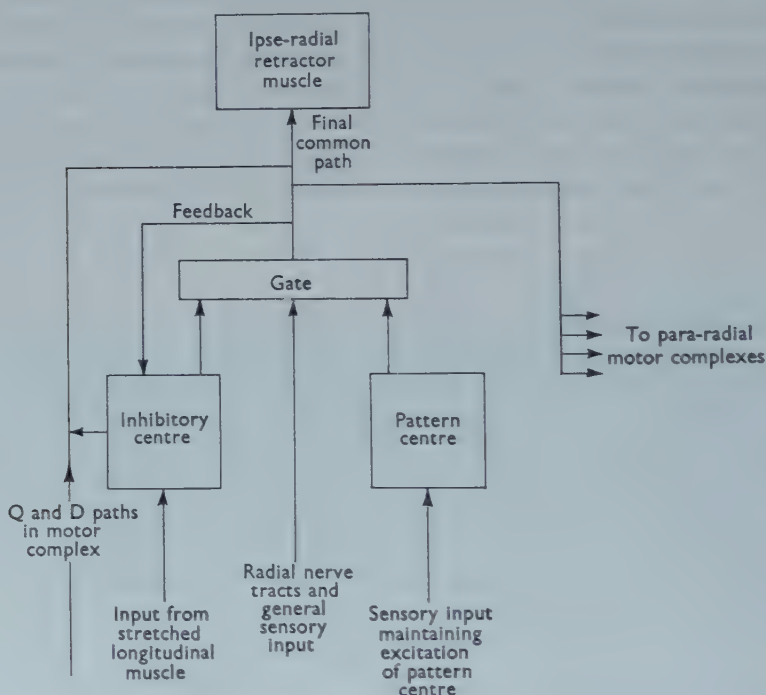


Fig. 14. Schematic representation of possible relations within the motor complex of a retractor muscle.

Impulses arising as a result of stretching the longitudinal muscle can enhance the excitation of the inhibitory centre so that all activity is prevented (Fig. 5). When the resultant inhibition is only partial the duration of the slow contractions is prolonged, suggesting inadequate feed-back to inhibit the response rapidly. At the same time the frequency with which these contractions occur falls (Fig. 6), which may be due to the local and longitudinal inhibitions summing; the level of local inhibition must now fall lower than normally before excitation impinging upon the gate will allow discharge of the pattern centre. It is, however, clear that many different and more elaborate 'schemes' to explain the present observations may be developed.

The major role of the retractor muscle of *Cucumaria* is that of drawing in the tentacular crown. This is a complex pattern of activity involving several muscles

and, so far, we have examined only one element of this pattern. This slow contraction appears, however, to be a 'fixed action pattern' and shows some of the properties characteristic of endogenous movements (Lorenz, 1950) of higher forms such as fishes and birds. Since it can occur 'spontaneously', it is not a simple reflex response to external stimuli but can be regarded as showing 'internal motivation' after the manner of an instinctive movement. Spontaneous activity of a retractor-muscle preparation may be regarded, albeit crudely, as 'vacuum activity'. A phenomenon resembling the dissipation of action specific energy following the performance of a consummatory act is also shown. This can be seen in a preparation in which the general level of excitation is low, as judged by the lack of spontaneous activity; in such a preparation activity in response to external stimulation is followed by a refractory period. These slow contractions thus show on the motor side parallels with phenomena attributed to the accumulation and discharge of 'action specific energy' in instinctive patterns of higher forms. On the sensory side, however, the difference is vast. In *Cucumaria* completely unspecific stimulation, such as light or touch, will elicit the response. This is indeed hardly remarkable, for the complex patterns of releaser stimuli associated with innate responses in higher forms can only occur when there are the exteroceptors capable of pattern discrimination and such are absent in *Cucumaria*. Nevertheless, the similarities of motor behaviour which we have emphasized suggest that there may be here, in simple form, the type of unit upon which more complex innate patterns are built; that the activity of such units is capable of a relatively simple formal explanation which introduces no novel principle of nervous activity and more especially, need not involve the idea of the slow accumulation of excitation or 'nervous energy', and that further study of this and similar activities, especially in sedentary animals where perceptual complications do not enter, may well assist our comprehension of the more elaborate activities of more active animals.

SUMMARY

1. In the apparent absence of any immediate external stimulation the retractor muscles of *Cucumaria* show slow contractions which take 1-2 min. to develop fully. These contractions develop the maximal possible tension and are of an all-or-nothing character.

2. The contractions are neurogenic in origin, ceasing if the retractor motor complex is destroyed and being only very rarely shown by a preparation in which the motor complex is de-afferented.

3. The slow contractions may be partially or fully inhibited by stretching the longitudinal muscle.

4. Slow contractions may be released by electrical stimulation of the radial nerve. The threshold of stimulation is higher than that for the previously described 'quick' and 'delayed' responses.

5. The slow contractions released by electrical stimulation are normally maximal, but partial slow contractions may be released in certain conditions. Slow contractions are not more frequently released by repetitive stimulation of the radial nerve.

6. The occurrence of a slow contraction modifies the characteristics of the quick and delayed responses.

7. When the activity of all five retractor muscles is recorded, co-ordinated slow responses may be observed; in such responses excitation appears to spread from one radius and to be conducted independently to the other four.

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FACTORS CONTROLLING THE CHANGE OF SHAPE OF CERTAIN NEMERTEAN AND TURBELLARIAN WORMS

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(Received 3 March 1958)

(With Plate 12)

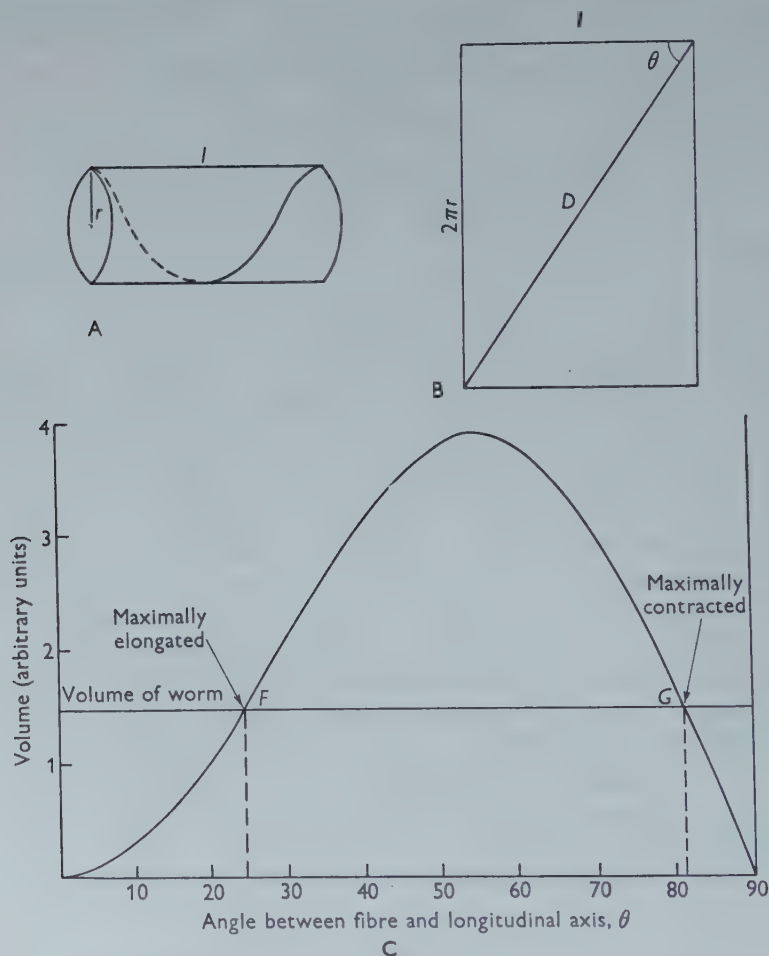
INTRODUCTION

Two groups of free-living, acoelomate worms, the nemerteans and turbellarians, are noted for their ability to change their shape. The role of circular and longitudinal muscles in producing these changes are well known, but since these worms are not invested by a cuticle, it is not immediately apparent what limits the changes of shape. Obviously the worms are not indefinitely extensible and compressible. Cowey (1952) discovered that the epidermal basement membrane of the nemertean *Amphiporus* is composed of regularly disposed, inextensible fibres arranged in alternate left- and right-handed geodesic helices running around the body of the animal (Text-figs. 1A; Pl. 12, figs. B, C and D). Although the fibres themselves are inextensible, changes of length of the body, or parts of the body, of the worm are permitted by a change in the angle between intersecting elements in the lattice formed by the fibres, just as in the extension and retraction of lazy tongs or a garden trellis. Cowey demonstrated the way in which these fibres set limits to the change in length of *Amphiporus*. A similar arrangement of fibrillar structures occurs in the cuticle of nematodes and functions in fundamentally the same way, although the nematode system is a highly specialized one (Harris & Crofton, 1957). In this paper we have considered the potentialities of this fibre system and the way it operates in a variety of turbellarians and nemerteans.

In considering the fibre system alone there is a simple relationship between the inclination of the fibres to the longitudinal axis and the length of the system. So for any particular orientation of the fibres the volume depends upon the cross-sectional area. This is greatest when the cross-section is circular, though it may be less if the cross-section is flattened to an ellipse and this can happen without any change in the length of the system, the orientation of the fibres, or the length of the perimeter of the cross-section. As the inclination of the fibres changes, so does the total volume the system can contain. Assuming a circular cross-section throughout, the volume varies as

$$V = \frac{D^3 \sin^2 \theta \cos \theta}{4\pi}, \quad (\text{see Eqn. 2, Appendix})$$

where D is the length of a single turn of the geodesic fibre bounding the system and θ is the angle between the fibre and the longitudinal axis. This relationship is illustrated in Text-fig. 1C and it will be seen that there are two limiting positions. The volume approaches zero as θ decreases and the system elongates to become a



Text-fig. 1 A. A unit length of a cylindrical 'worm' bounded by a single turn of the geodesic fibre system. (Fibres running in the opposite sense have been omitted for clarity.) B. The same unit length of worm slit along the top and flattened out. C. The curve represents the theoretical relationship between the volume contained by the fibre system and the inclination of the fibres to the longitudinal axis. The horizontal line represents the actual and constant volume of the nemertean *Amphiporus lactifloreus*. It intersects the curve at F and G which are the limiting positions of elongation and contraction, respectively, for that species.

long, thin thread, and the length approaches D . The other limit, when the volume again approaches zero, is when $\theta = 90^\circ$; the system is then reduced to a thin disk with a circumference equal to D . At an intermediate point, where $\tan^2 \theta = 2$, or $\theta = 54^\circ 44'$, the volume contained by the system is at a maximum.

When the antagonistic longitudinal and circular muscles operate on a fluid skeleton it is necessary that the volume should remain constant in order to produce reversible changes of shape (see, for example, Chapman, 1950); and, in fact, in coelomate worms where the coelomic fluid functions as a fluid skeleton, precautions are taken to ensure that none leaks through the nephridia or dorsal pores during the pressure changes that accompany changes in length (Chapman & Newell, 1947; Newell, 1950). When we consider the functioning of the fibre system in a worm it is obvious that if the worm had that volume which is the greatest the fibre system can contain, it would be incapable of any change in length, for to do so would involve a reduction in its volume. This condition almost obtains when a nemertean is full of ripe gonads. However, the volume represented in Text-fig. 1C and Eqn. 2, Appendix, is the maximal and limiting volume for any particular length and orientation of the fibres. The system can always contain less than this volume if the cross-section is elliptical instead of circular. The limits of extension and contraction of the worm are set by the points at which the actual volume of the worm is equal to the greatest volume the system can contain at that particular length and value of θ . At these points the worm has a circular cross-section; to extend or contract beyond these points would involve a diminution of volume and that is precluded. Between these points the volume of the worm is less than the limit set by the fibre system and the cross-section is elliptical. The horizontal line in Text-fig. 1C represents the volume of the littoral nemertean *Amphiporus lactifloreus*, and its intersection with the curve at *F* and *G* represent the limits of extension and contraction, respectively, limits which the worm almost reaches in practice and which are determined by the fibre system and the volume of the worm (Cowey, 1952).

In the nematodes the basic properties of the helical fibres in the cuticle are the same as those of the nemertean basement membrane. However, the entire system functions in a highly specialized way in nematodes as Harris & Crofton (1957) have recently demonstrated in their study of *Ascaris*. The chief peculiarities of this nematode (and presumably of other nematodes) are (i) the very high internal hydrostatic pressure, which is opposed by the thick cuticle, and (ii) the existence of only longitudinal muscles in the body wall. As a result of the high internal pressure the cross-section is always circular, although the inclination of the fibres to the longitudinal axis is about 75° . At this point on the curve in Text-fig. 1C a contraction of the longitudinal muscles causing an increase in θ , involves a reduction in the volume the fibre-system can contain, or, since the volume of the worm is itself invariable, an increase in turgor pressure. It is this which acts antagonistically to the longitudinal muscles and accounts for the absence of circular muscles. The nematode system can function only over the right-hand half of the curve in Text-fig. 1C. A worm possessing only circular muscles in the body wall could function only over the left-hand half of the curve. Nemerteans and, indeed, all worms other than nematodes and Nematomorpha possess both circular and longitudinal muscles, and the system functions over both right and left halves of the curve within limits set by the volume of the animal and the dimensions of the fibre system.

Helical bounding systems such as occur in the basement membrane of *Amphiporus* and the cuticle of *Ascaris* may be quite widespread, if not general, in soft-bodied, worm-like animals (Picken, Pryor & Swann, 1947). We have, therefore, considered the theoretical potentialities of such a system as they affect the change of length and shape of worms, and have compared the theoretical findings with the actual performance of a number of nemerteans and turbellarians. These worms possess both circular and longitudinal muscles in the body wall and are not constrained by a cuticle. The way in which these muscles can produce changes in body shape within limits set by the fibre system can thus be studied with a minimum of complicating factors.

MATERIALS AND METHODS

The following turbellarians and nemerteans have been selected from a wide range of ecological situations to exhibit a variety of body shapes.

Nemerteans

Amphiporus lactifloreus (Johnston), 10 specimens, marine, littoral.

Lineus gesserensis (Müller), 10 specimens, marine, littoral.

L. longissimus (Gunnerus), 1 specimen, marine, littoral.

Cerebratulus lacteus (Leidy), 2 specimens, marine, burrowing and swimming form.

Malacobdella grossa (O. F. Müller), 10 specimens, marine, parasitic on the gills of lamellibranchs.

Geonemertes dendyi Dakin, 10 specimens, terrestrial.

Turbellarians

Dendrocoelum lacteum Oersted, 10 specimens, fresh-water streams.

Polycelis niger Ehrenberg, 10 specimens, fresh-water streams.

Rhynchodemus bilineatus (Mecznikow), 2 specimens, terrestrial.

The worms were completely anaesthetized in magnesium chloride sea water, or in 5% pure ethyl alcohol in pond water, and then fixed in Zenker-formol, embedded in celloidin-paraffin and sectioned in the plane of the basement membrane or transversely. They were stained by Wilder's (1935) silver impregnation technique for reticulin fibres.

From the following analysis, it will be seen that the quantities, length and volume, refer to the length and volume of a unit piece of worm which is invested by a single turn of the geodesic helical fibre. Therefore not all the quantities that appear in the equations can be directly measured.

A completely anaesthetized worm takes up the position where $\theta = 54^\circ 44'$. This is probably due to a tendency for the semi-fluid constituents of the worm to flatten out as much as possible, and, as we have seen, the greatest flattening that is possible is at the position where there is the greatest discrepancy between the actual volume of the worm and the maximum volume the system can contain. If an anaesthetized worm is passively stretched and then released it returns to this equilibrium, or relaxed, length so that this is a position of great stability which can be

attained simply and reliably and one in which measurements can be made with the greatest accuracy. It is impossible to measure length and volume of a unit section of the worm directly, but they are related to, and can be deduced from, the degree of flattening that occurs in the relaxed position. In fact, the ratio of actual volume to the greatest volume the system can contain is given by

$$\frac{2n_r}{n_r^2 + 1}, \quad (\text{see Eqn. 5, Appendix})$$

where n_r is the ratio of the length of the major to the minor axis of the elliptical cross-section of the worm in the relaxed position. If n_r is known, a horizontal line can be drawn on the curve, as in Text-fig. 2C, and its points of intersection with the curve give the theoretical maximum and minimum length of the worm.

The fully extended position can be reached by passively stretching an anaesthetized worm. Once that position is reached considerably greater tension must be applied to tear the tissues. The worm can then be fixed in the fully extended state. The maximally contracted position can be obtained by fixing an unanaesthetized worm directly. Obviously this is a much less accurately determinable position.

From measurements made on the fully extended, fully contracted and relaxed animals, it is possible to calculate all the parameters necessary for a comparison of actual performance with the theoretical predictions.

THE STRUCTURE OF THE BASEMENT MEMBRANE

In all nine species of nemerteans and turbellarians studied the structure of the epidermal basement membrane is the same and answers to the description of that of *Amphiporus lactifloreus* given by Cowey (1952). There are alternating layers of reticulin fibres arranged in left- and right-handed geodesic helices. All the worms possess both longitudinal and circular muscles in the body wall, and the fact that some nemerteans have a second layer of longitudinal muscles is irrelevant to the following argument.

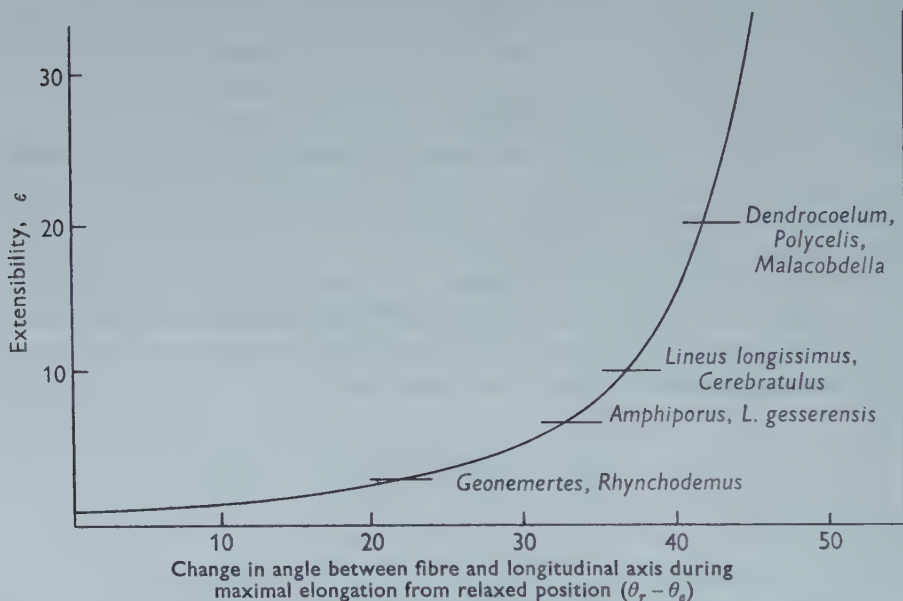
THE EXTENSIBILITY OF THE WORMS

The extensibility, ϵ (ratio of maximum to minimum lengths), can now be worked out. Text-fig. 2 shows the relationship between the change in the inclination of the fibres on extension from relaxed length to maximum length ($\theta_r - \theta_e$) and the extensibility. The extensibilities which the species under consideration would achieve if the fibre system alone set a limit to their changes in length are marked on the curve. Considerable change in the orientation of the fibres is necessary before large extensibilities are attained, but once a certain point is reached enormous extensibilities are theoretically possible for slight additional changes in the fibre system. The actual extensibilities of the worms can be measured only approximately. The actual and theoretical extensibilities are compared in Table 1.

The performance of the worms with low theoretical extensibilities agrees quite well with the predicted value, those with moderate powers of extension and con-

traction less well, but there is an enormous discrepancy between the theoretical and actual values in those species for which very large extensibilities are predicted.

Rhynchodemus bilineatus and *Geonemertes dendyi* have low theoretical extensibilities so that they never depart far from the relaxed position and show a close approximation to a circular cross-section at any length. Their actual extensibility is the same as the theoretical (Text-fig. 3), so that the geodesic fibres are the operative factor in limiting changes of length of these worms.



Text-fig. 2. The theoretical relationship between the extensibility (ratio of fully extended to fully contracted lengths) and the change in the inclination of the fibres from that at the relaxed position as the worm elongates. The theoretical extensibilities of some turbellarians and nemerteans are marked on the curve.

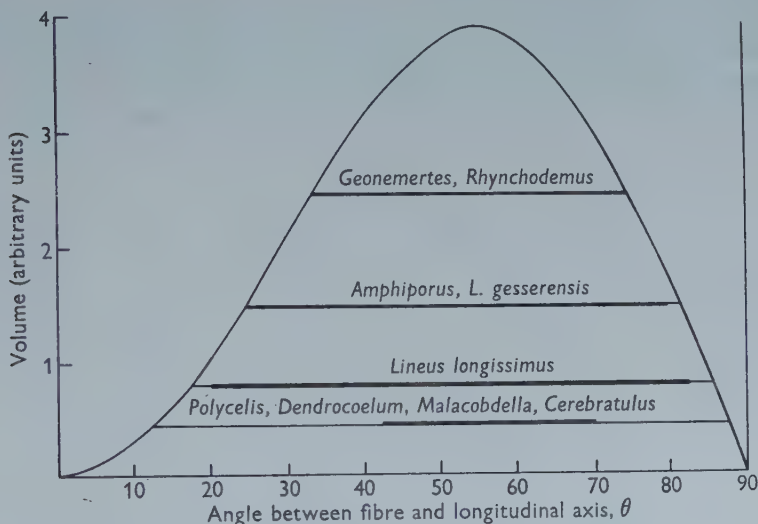
Table 1

Species	Theoretical extensibility	Actual extensibility
<i>Rhynchodemus bilineatus</i>	3	3
<i>Geonemertes dendyi</i>	3-4	3-4
<i>Amphiporus lactifloreus</i>	6-7	5-6
<i>Lineus gesserensis</i>	6-7	5-6
<i>L. longissimus</i>	> 10	< 9
<i>Cerebratulus lacteus</i>	> 10	2-3
<i>Dendrocoelum lacteum</i>	> 20	2-3
<i>Malacobdella grossa</i>	> 20	2-3
<i>Polycelis niger</i>	> 20	2

Amphiporus lactifloreus, *Lineus gesserensis* and *L. longissimus* all have an actual extensibility which falls a little short of the theoretical limits of the system. This is indicated by their not quite attaining a circular cross-section at the minimum length

or at both maximum and minimum lengths. The former is the case with *Amphiporus* and *Lineus gesserensis*, the latter with *L. longissimus* (Text-fig. 3). Longitudinal sections of all three species cut from worms that were completely contracted show that the basement membrane is thrown into regular folds (Pl. 12, fig. D), the folds running transversely around the animals. Transverse sections of *L. longissimus* at maximum length also show a regular folding of the basement membrane, but in this case it is a longitudinal folding. Examination of the sections gives the impression that the membrane is folded in order to preserve the area necessary for the support of the epidermal structures resting on it. Whatever the cross-sectional shape of the worm, the surface area is proportional to

$$\sin \theta \cos \theta, \quad (\text{see Eqn. 6, Appendix})$$



Text-fig. 3. The relationship between the volume contained by the fibre system and the inclination of the fibres (as in Text-fig. 1C) on which are superimposed the actual volumes of various nemerteans and turbellarians (fine horizontal lines). The heavy lines show the range over which changes in length take place, indicating where the worms do not reach the limiting positions set by the fibre system (exaggerated for clarity).

and in Text-fig. 4 the relation between the surface area and θ is illustrated. The portion of the curve XY shows how the surface area of *Amphiporus* changes as the worm contracts from maximum to minimum length. The surface area at minimum length is a good deal less than at maximum length and apparently is not sufficient to contain the bases of all the epidermal cells. To some extent this is offset by the folds in the basement membrane which provide the extra area required, but evidently it is insufficient to counteract this effect completely. Thus, in both *Amphiporus* and *Lineus gesserensis*, the maximum length is determined by the fibre system but the minimum length is determined by the minimum surface area which will still accommodate all the epidermal cells. The compression of the epidermis sets a limit to both maximum and minimum lengths of *L. longissimus*, but at the

maximum length the folds are parallel to the longitudinal axis because the circular muscles are contracted and the pressures act transversely in the plane of the basement membrane, i.e. around the worm.

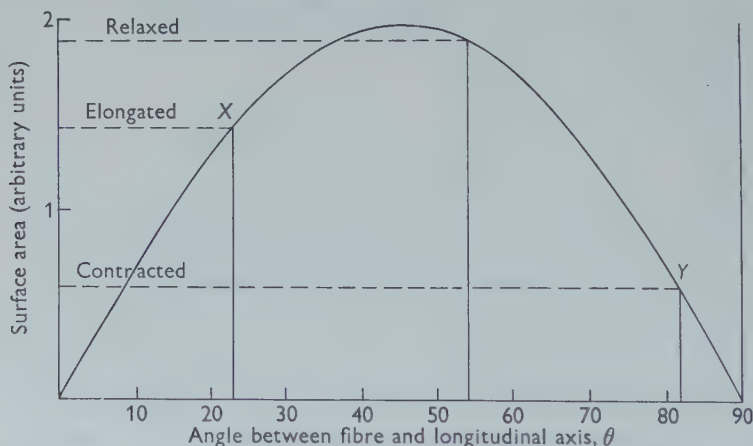
The other four species, *Cerebratulus lacteus*, *Malacobdella grossa*, *Polycelis niger* and *Dendrocoelum lacteum*, all fall very far short of attaining their theoretical extensibilities (Text-fig. 3). The factors responsible for this include:

(1) The longitudinal reticulin fibres in the longitudinal muscle layers which limit the maximum length.

(2) The reticulin fibres in the circular muscle layers (Pl. 12, fig. C), which limit the circumference, and so the minimum length.

(3) The dorso-ventral muscles which preserve the flattened form and so restrict very markedly the changes of length.

(4) The thickness of the body-wall musculature in *Cerebratulus*, a swimming nemertean. In this discussion it has been assumed that the thickness of the muscle layers is negligible. Obviously, if there are large muscle blocks which are not readily deformed, as in *Cerebratulus*, changes in shape are greatly limited.



Text-fig. 4. The theoretical relationship between the surface area of a unit length of worm and the inclination of the fibres. The points X and Y are the theoretical limiting positions of elongation and contraction, respectively, of *Amphiporus lactifloreus*.

CHANGES IN CROSS-SECTIONAL SHAPE

Attention has already been drawn to the fact that ideally the worm is circular in cross-section at the limiting positions of maximum extension and contraction and that it progressively flattens towards the relaxed position.

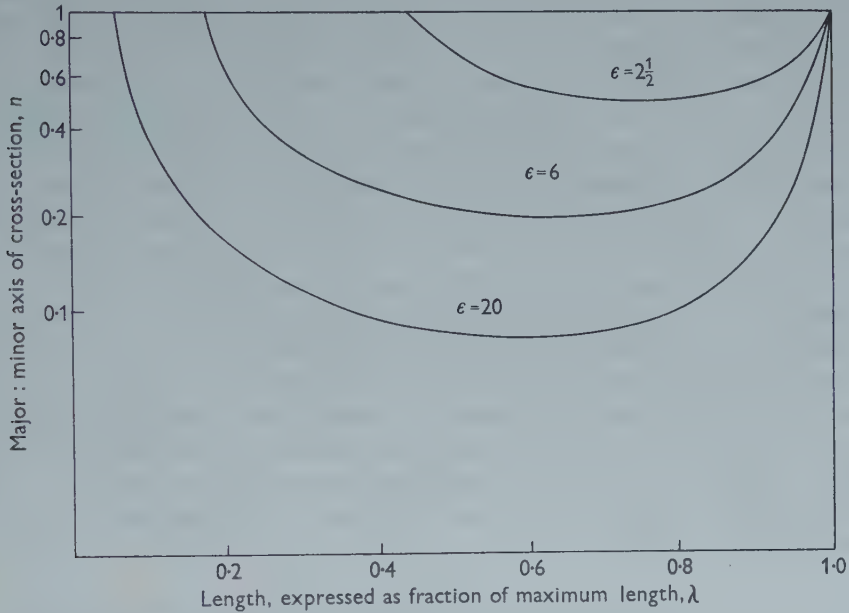
If n is the ratio of major to minor axes of the elliptical cross-section and λ the ratio of the length at any position to the theoretically maximum length,

$$n = \frac{\lambda(\sec^2 \theta_e - \lambda^2) - \sqrt{[\lambda^2(\sec^2 \theta_e - \lambda^2)^2 - \tan^4 \theta_e]}}{\tan^2 \theta_e}.$$

(See Eqn. 11, Appendix)

This expression has been evaluated for three values of the parameter θ_e (the value of θ at the maximally extended position), corresponding to the extensibilities of the three groups of worms in Table 1, and n is shown plotted against λ in Text-fig. 5. The degree of flattening is, of course, directly related to the extensibilities of the worms.

There are a number of consequences of the change of shape of the body, quite apart from the extensibility, which may be related to the ecology of the three groups of worms.



Text-fig. 5. The flattening of the body as worms pass from theoretical maximum to theoretical minimum length for worms of different extensibilities corresponding to the three groups of species in Table 1.

(1) Those which have slight extensibilities and do not flatten appreciably are terrestrial and move on mucus which they secrete. Conservation of water must therefore present a problem to these animals. Loss by evaporation is reduced to a minimum because the surface area: body volume ratio is minimal when the cross-section is circular. A circular cross-section could be achieved if the worms were fully contracted or fully extended, or if, as in this case, the worms are almost circular in cross-section at the relaxed position and the extensibility is very low. The latter is the more economical and in fact obtains in *Rhynchodemus* and *Geonemertes*.

(2) Those which flatten to a moderate extent and have a considerable extensibility are marine crawling forms and are therefore not concerned with water conservation. Crawling is by means of cilia when the worm is relaxed and the body flattened, and the greatest surface area is presented to the ground.

(3) Those which are markedly flattened but have low extensibilities have ecological or mechanical requirements which demand a permanently flattened form even at the expense of extensibility. *Malacobdella* lives in the mantle cavity of *Cyprina* and other lamellibranchs. It is provided with a sucker in order to maintain its position when the host closes the valves of its shell and shoots a stream of water through its siphons. Its flattened form is obviously necessary for it to live between the gill lamellae or between the gills and the visceral mass of the bivalve. *Cerebratulus* requires a flattened body for effective swimming. The powerful and bulky longitudinal muscles, which are also needed, prevent the worm from realizing the powers of changing its shape which such a body-form otherwise confers upon it. The turbellarians *Polycelis* and *Dendrocoelum* are permanently flattened probably for physical reasons. The mass of an animal generally increases as the cube, the surface area as the square of its linear dimensions. The locomotory efficiency of animals that move by cilia is a function of the surface area, so that at some stage the mass becomes too great to be transported by cilia and a fairly low limit is set to the size the animal can reach. But if the thickness of the animal is the same whatever its size, as is approximately true of the flatworms, mass and surface area both increase as the square of the linear dimensions and no limit is set to the size they can attain. In fact some turbellarians reach comparatively large sizes and still move by ciliary action. A very flat, thin body-form is therefore a mechanical necessity for these worms if they are to move by cilia. A contributory ecological factor which may account for the flattening of some turbellarians, though probably not appreciably for that of *Polycelis* and *Dendrocoelum*, is that they live in fast-flowing streams or in areas of strong tidal currents that might wash them off the stones on which they crawl. A flattened form reduces the likelihood that they will be swept off the substratum.

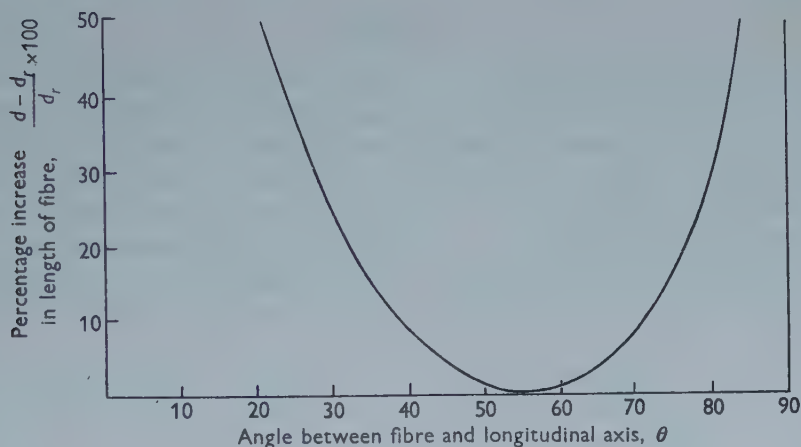
ELASTICITY OF THE FIBRES

The reticulin fibres of the basement membrane of *Amphiporus* were found to be inextensible (Cowey, 1952), at least to the extent that they could be snapped on micro-dissecting needles without apparent change in length. The fibres composing the comparable structure in the cuticle of *Ascaris* were assumed to be inextensible by Harris & Crofton (1957). However, it seems possible that some slight elasticity of the fibres might be sufficient to account for the observed extensibilities of the whole worms, particularly of those which do not show marked powers of extensibility.

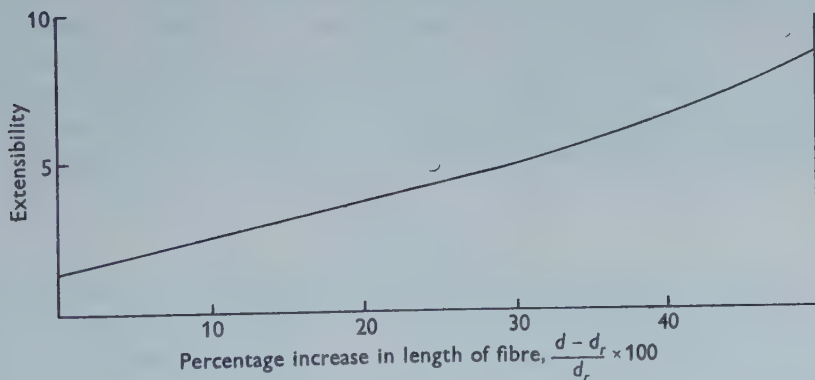
If the cross-section is assumed to remain circular and the fibres to be extensible, they are shortest in the relaxed position where $\theta = 54^\circ 44'$ and elongation or contraction of the worm involves stretching them. The limits of extension and contraction would be set by the elastic limit of the fibres. If d is the length of the fibres when they are stretched to the elastic limit and d_r the resting length of the fibres,

$$\frac{d - d_r}{d_r} = \left(\frac{\cos \theta_r - \cos^3 \theta_r}{\cos \theta - \cos^3 \theta} \right) - 1. \quad (\text{See Eqn. 13, Appendix})$$

The relationship between $(d-d_r)/d_r$, which is the fractional increase in the length of the fibres, and θ is illustrated in Text-fig. 6. For any value of $(d-d_r)/d_r$, there are two values of θ which correspond to the positions of maximum and minimum length, and from these we can calculate the extensibilities of the worms for any value of $(d-d_r)/d_r$. This is illustrated in Text-fig. 7. To produce an extensibility of $2\frac{1}{2}$, the fibres would have to stretch 10% of their resting length, and to produce



Text-fig. 6. Percentage increase in the length of the fibres, assuming them to be elastic and the volume of the system constant, as the inclination of the fibres changes.



Text-fig. 7. The relationship between the extensibility conferred on worms of constant volume bounded by an elastic fibre system and the elasticity of the fibres.

an extensibility of $3\frac{1}{2}$, more than 20%. It is obvious that a slight extensibility of the geodesic fibres will not account for even the smallest extensibilities that have been observed in the worms. However, it is interesting to apply the results of this discussion to a consideration of the diagonal muscles which are found developed to a certain extent in nemerteans (Bürger, 1895) and are very highly developed in

some turbellarians (Lang, 1884). Synergic contraction of the diagonal musculature will always result in the animal returning to the resting length. This will happen whether the animal is at a length greater or less than the resting length when the contraction of the diagonal muscles takes place. Their greater development in turbellarians is obviously correlated with the flattened form of these worms and prevent too great a departure from the relaxed position where the flattening is maximal.

DISCUSSION

A geodesic helical boundary system of fibres such as we have described provides an elastic tissue on which the epidermal cells rest, although it is itself composed of inelastic elements (the reticulin fibres). The system sets an over-all limit to changes in shape, though often other factors supervene and impose closer limits on the flexibility of the worm. Some of these factors, such as the existence of dorso-ventral and diagonal muscles in turbellarians, are clearly adaptive; others such as the compression of the epidermal cells or the existence of a bulky body-wall musculature, are accidental mechanical consequences of the morphology of the worms.

A helical fibre of this sort is known to occur in the basement membrane of only the nemerteans and turbellarians, but a similar system is found in the nematode cuticle and possibly also in the cuticle of annelids (Picken *et al.* 1947; Harris & Crofton, 1957) and may well be found to be a feature of the epidermal basement membrane and the cuticle of all worm-like animals. Clearly, the fundamental dynamic properties of such a fibre system are the same, whether it is internal or external to the epidermis, for in all cases it represents a bounding skeletal system which at once permits a flexibility to the body wall and also sets limits to that flexibility. No worm which possesses a cuticle has anything like the extensibility of some species of nemertean, possibly because the non-cellular matrix within which the cuticular fibre system lies has only very limited elastic properties.

SUMMARY

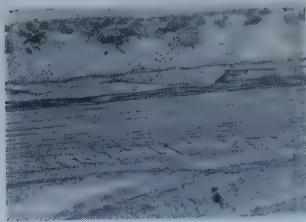
1. Nemerteans and turbellarians have an inextensible fibre system around them in the form of a lattice of left- and right-handed spirals. The effect of this system on the change of shape on these worms has been analysed theoretically and compared with the observed behaviour of nine species of turbellarian and nemertean from widely differing habitats.

2. The following theoretical relationships have been studied:

(a) Variation of the angle between the geodesics and the longitudinal axis of the worm during changes in length, and the role of the fibre system in limiting changes in length of the animal.

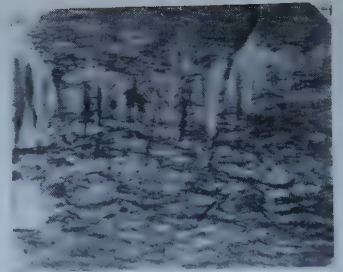
(b) The change in cross-sectional shape during changes in length.

(c) The extension of the fibres and the extensibility of the worms, assuming the fibres of the lattice to be elastic.



A

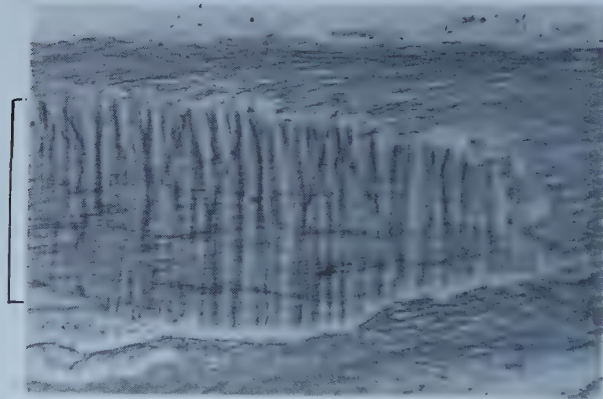
100 μ



B

100 μ

Circular muscle with argyrophil fibres of myoseptum showing through

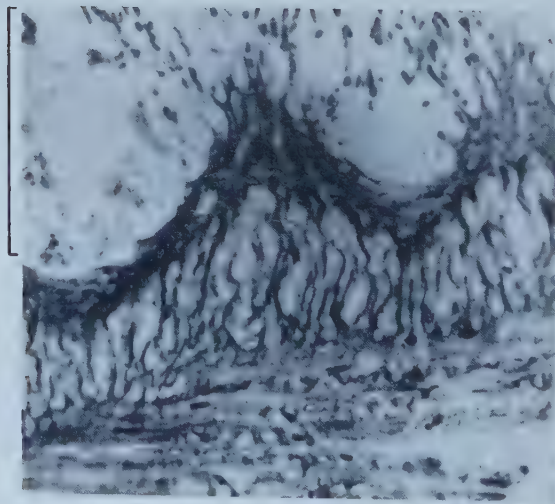


C

100 μ

Basement membrane

Epidermis



D

100 μ

Basement membrane

Longitudinal muscle layer

Circular muscle layer

CLARK AND COWEY—FACTORS CONTROLLING THE CHANGE OF SHAPE OF CERTAIN NEMERTEAN AND TURBELLARIAN WORMS

(Facing p. 743)

3. The species investigated conform with the theoretical predictions to varying degrees and have been grouped accordingly:

(a) *Geonemertes dendyi* and *Rhynchodemus bilineatus* have low extensibilities and fit the prediction well. They are nearly circular in cross-section at all lengths as a result of their low extensibility and this is related to their terrestrial habit and need for water conservation.

(b) *Amphiporus lactifloreus*, *Lineus gesserensis* and *L. longissimus* are moderately flattened in the relaxed position and have extensibilities between 6 and 10. They are marine crawling forms using cilia for locomotion and so must present a fairly large ciliated surface to the substratum. The fibre system does not limit contraction; the compression of the epithelial cells causes the observed extensibilities to fall a little short of the theoretical values.

(c) *Cerebratulus lacteus*, *Malacobdella grossa*, *Polycelis nigra* and *Dendrocoelum lacteum* are very flattened forms and have very high theoretical extensibilities, but very low observed ones. The factors causing this are the thickness of the body-wall musculature (*Cerebratulus*), the limiting effect of longitudinal and circular reticulin fibres in the muscle layers, and the presence of dorso-ventral and diagonal muscles. Their flattened form is correlated with ecological factors (with swimming in *Cerebratulus*, with its parasitic life in the mantle of bivalves in *Malacobdella*) or with physical ones in turbellarians where a permanently flattened form is necessary for these worms to move by ciliary action.

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EXPLANATION OF PLATE

- A. Transverse section of the body wall of *Lineus gesserensis*. (Zenker-formol; Heidenhain's Azan.)
 B. Section in the plane of the basement membrane of *Amphiporus lactifloreus* (Zenker-formol; Wilder's technique.)
 C. Slightly oblique section through the basement membrane of *Amphiporus lactifloreus* showing also the argyrophilic fibres of the myoseptum. (Zenker-formol; Wilder's technique.)
 D. Transverse section of *Lineus gesserensis* to show the folding of the basement membrane when the worm is maximally contracted. (Zenker-formol; Wilder's technique.)

APPENDIX

Symbols used in the appendix

- A Surface area of an element invested by one turn of the geodesic.
 a Semi-major axis of the cross-section.
 b Semi-minor axis of the cross-section.
 a_r Value of a at relaxed length.
 b_r Value of b at relaxed length.
 D Constant length of one turn of the inextensible geodesic fibre.
 d Length of one turn of the elastic geodesic fibre.
 d_r Length of d at relaxed length.
 l Length of an element investigated by one turn of the geodesic.
 l_e Value of l at maximally extended position.
 l_c Value of l at maximally contracted position.
 n Ratio of a/b .
 n_r Ratio of a_r/b_r .
 r Radius of circular cross-section.
 V Volume of an element invested by one turn of the geodesic.
 ϵ Extensibility, ratio of l_e/l_c .
 θ Angle between the fibres of the lattice and the longitudinal axis.
 θ_c Value of θ at minimum length.
 θ_e Value of θ at maximum length.
 θ_r Value of θ at relaxed length.
 λ Value of ratio l/l_c .

(1) *Change in length and extensibility*

Consider a length of worm invested by a single turn of the geodesic fibre system (Text-fig. 1A, B). The fibres are assumed to be inextensible and the cross-section to remain circular

$$l = D \cos \theta, \quad (1)$$

$$r = \frac{D \sin \theta}{2\pi},$$

$$V = \pi r^2 l.$$

Then substituting for r and l

$$V = \frac{D^3 \sin^2 \theta \cos \theta}{4\pi}. \quad (2)$$

The maximum value of V is reached when $dV/d\theta$ is zero, i.e. where $\tan \theta = \sqrt{2}$ or $\theta = 54^\circ 44'$, and this point represents the equilibrium position of the system (Text-fig. 1C).

The extensibility, ϵ , is the ratio of the extended to the contracted lengths

$$\epsilon = \frac{l_e}{l_c} = \frac{D \cos \theta_e}{D \cos \theta_c} = \frac{\cos \theta_e}{\cos \theta_c}. \quad (3)$$

Since the volume of the worm is constant and the same at the two points θ_e and θ_c , from equation (2) we have

$$\sin^2 \theta_c \cos \theta_c = \sin^2 \theta_e \cos \theta_e. \quad (4)$$

From equations (3) and (4) we may calculate ϵ for any value of θ_e by substituting the appropriate value of θ_e in equation (4), solving the resulting cubic equation in $\cos \theta_c$ and substituting the result in equation (3). The relationship between θ_e and ϵ is illustrated in Text-fig. 2.

The values of θ_e and θ_c cannot be measured readily because of the folding of the basement membrane which occurs in some species when the worms are fully extended or contracted, but the values can be deduced if the ratio of major to minor axes of the cross-section at the relaxed position is known.

The perimeter of the elliptical cross-section (Text-fig. 8 A, B) is approximately*

$$2\pi \sqrt{\left[\frac{1}{2}(a_r^2 + b_r^2)\right]} = 2\pi \sqrt{\left[\frac{1}{2}a_r^2 \left(\frac{n_r^2 + 1}{n_r^2}\right)\right]},$$

and the volume of an element of length l is

$$\pi a_r b_r l = \frac{\pi a_r^2 l}{n_r}.$$

The greatest volume the system can contain at the relaxed position is that when the cross-section is circular, with radius r . The actual volume of an element of the worm need not be so great, in which case the cross-section is flattened to an ellipse with the same perimeter as the circle. Thus:

$$2\pi r = 2\pi \sqrt{\left[\frac{1}{2}a_r^2 \left(\frac{n_r^2 + 1}{n_r^2}\right)\right]}.$$

The greatest volume the system can contain is

$$\pi r^2 l = \pi \frac{1}{2} a_r^2 \left(\frac{n_r^2 + 1}{n_r^2}\right) l,$$

substituting for r from the previous equation. So that the ratio of the actual volume of the element at relaxed length to the greatest volume the system can contain is

$$\frac{\pi(a_r^2/n_r) l}{\pi \frac{1}{2} a_r^2 [(n_r^2 + 1)/n_r^2] l} = \frac{2n_r}{n_r^2 + 1}. \quad (5)$$

The theoretical extensibility of the worm can be read off the graph in Text-fig. 1 C after a horizontal line has been drawn representing the actual volume of the element, or it can be calculated by manipulating equations (2) and (3).

* This expression is accurate for nearly circular figures, but overestimates the value of the perimeter if the ellipse is markedly flattened. If $a = 10b$, the error is nearly 10%.

(2) *Changes in surface area*

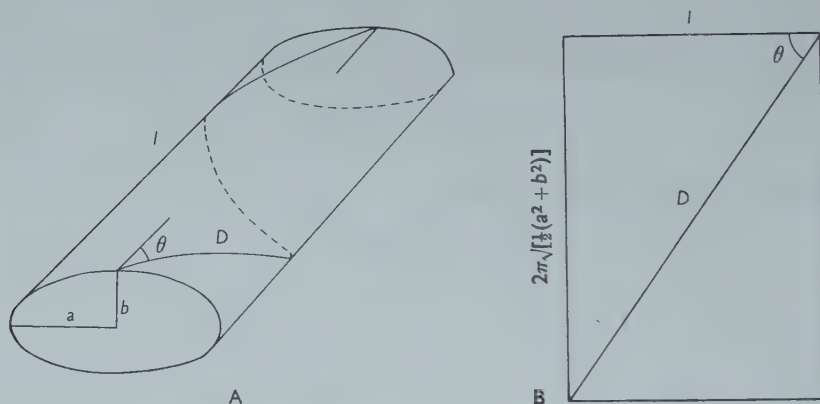
The length of an element is $D \cos \theta$ and the circumference, whatever the cross-sectional shape, is $D \sin \theta$ (see Text-fig. 1 B), so that the surface area

$$A = D^2 \sin \theta \cos \theta,$$

and since D , the length of the fibre, is constant

$$A \propto \sin \theta \cos \theta. \quad (6)$$

The relationship between A and θ is illustrated in Text-fig. 4, where A is plotted in arbitrary units.



Text-fig. 8A. A unit length of a cylindrical 'worm' with an elliptical cross-section bounded by a single turn of the geodesic fibre system. B. The same unit length slit along the top and flattened out.

(3) *Change in cross-sectional shape*

The element is circular in cross-section at the limits of extension and contraction set by the geodesic fibres and elliptical at intermediate points. If this ellipse has semi-major and minor axes of a and b respectively, and the ratio of major to minor axes is n (see Text-fig. 8):

$$V = \pi a b l, \quad (7)$$

$$d^2 = l^2 + 2\pi^2(a^2 + b^2), \quad (8)$$

$$d = l_e^2 \sec^2 \theta_e. \quad (9)$$

Equation (7) becomes

$$V = \pi n b^2 l,$$

and eliminating d , a and b from equation (8) we have

$$l_e \sec^2 \theta_e = l^2 + 2\pi \frac{V}{\pi n l} (1 + n^2),$$

or

$$V = (l_e^2 \sec^2 \theta_e - l^2) \frac{n l}{2\pi(1 + n^2)}.$$

Now the volume of the element is constant so that

$$dV = \frac{n}{2\pi(1+n^2)} (l_e^2 \sec^2 \theta_e - 3l^2) dl + \frac{l}{2\pi} (l_e^2 \sec^2 \theta_e - l^2) \frac{(1-n^2)}{(1+n^2)^2} dn = 0,$$

i.e.
$$\frac{n}{2\pi(1+n^2)} (l_e^2 \sec^2 \theta_e - 3l^2) dl = \frac{l}{2\pi} (l_e^2 \sec^2 \theta_e - l^2) \frac{n^2-1}{(1+n^2)^2} dn.$$

This may be arranged in an integrable form:

$$\frac{l_e^2 \sec^2 \theta_e - 3l^2}{l(l_e^2 \sec^2 \theta_e - l^2)} dl = \frac{n^2-1}{n(n^2+1)} dn. \quad (10)$$

The constant of integration can be found by considering the maximally extended position. There $l = l_e$ and $n = 1$, since the cross-section is circular.

The complete integration of equation (10) is

$$\frac{n^2+1}{n} = 2\lambda \frac{(\sec^2 \theta_e - \lambda^2)}{\tan^2 \theta_e},$$

where $\lambda = l_e/l$. This is a simple quadratic equation in n , the roots of which are

$$n = \frac{\lambda(\sec^2 \theta_e - \lambda^2) \pm \sqrt{[\lambda^2(\sec^2 \theta_e - \lambda^2)^2 - \tan^4 \theta_e]}}{\tan^2 \theta_e}.$$

The two roots are reciprocals of each other and correspond to the conditions that $a/b = n$ or $b/a = n$, so that the only one which need be considered is

$$n = \frac{\lambda(\sec^2 \theta_e - \lambda^2) - \sqrt{[\lambda^2(\sec^2 \theta_e - \lambda^2)^2 - \tan^4 \theta_e]}}{\tan^2 \theta_e}. \quad (11)$$

The relationship between n and λ has been computed for three values of θ_e and is illustrated in Text-fig. 5.

(4) *The elasticity of the fibres*

The extensibility of a geodesic system in which the volume remains constant and the cross-section circular, but in which the fibres are elastic can be calculated as follows.

From Text-fig. 1:

$$V = \pi r^2 l,$$

$$d^2 = l^2 + 4\pi^2 r^2.$$

Therefore

$$d^2 = l^2 + \frac{4\pi V}{l};$$

or

$$V = \frac{d^2 l - l^3}{4\pi}. \quad (12)$$

Since $d = l \cos \theta$, equation (12) can be rearranged and written

$$d = \left(\frac{4\pi V}{\cos \theta - \cos^3 \theta} \right)^{\frac{1}{2}}.$$

This has a minimum value when $\theta = 54^\circ 44'$ as in the case considered in section 1 of the Appendix.

If d_r is the length of d at this minimum position, the fractional increase in the length of the fibre at any value of θ is

$$\frac{d-d_r}{d_r} = \left(\frac{\cos \theta_r - \cos^3 \theta_r}{\cos \theta - \cos^3 \theta} \right)^{\frac{1}{3}} - 1, \quad (13)$$

where θ_r is the value of θ at the relaxed length, ie. $\theta = 54^\circ 44'$. The relationship between $(d-d_r)/d_r$ and θ is illustrated in Text-fig. 6.

For any value of $(d-d_r)/d_r$, equation (13) is satisfied by the value of θ corresponding to maximum elongation and contraction of the worm. The extensibility

$$\epsilon = \frac{l_e}{l_c} = \frac{\cos \theta_e}{\cos \theta_c},$$

so that the extensibility can be determined for any value of the elasticity of the fibres. The relationship is illustrated in Text-fig. 7.

THE INORGANIC AND AMINO ACID COMPOSITION OF SOME LAMELLIBRANCH MUSCLES

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(Received 27 February 1958)

INTRODUCTION

The electrolyte composition of vertebrate striated muscle has been the subject of many investigations, and some of the principles governing the distribution of electrolytes between the muscle fibres and the plasma are fairly well understood. In comparison with the plasma the sarcoplasm of striated muscle contains a low concentration of sodium and chloride and a high concentration of potassium. This unequal distribution is a dynamic equilibrium maintained by the active extrusion of sodium from the cell. The distribution of potassium and chloride is very close to a Donnan equilibrium across the potential gradient produced by the sodium extrusion and by a concentration of indiffusible anions inside the fibres. Less is known about ionic equilibrium in invertebrate muscle, but a recent detailed investigation of the fibres of the muscles of *Carcinus maenas* suggests that the conditions are similar to those found in the vertebrates (Shaw, 1955). Gross analyses of *Loligo* muscle (Manery, 1939) would fit into the same picture if allowance is made for a small quantity, about 5%, of extracellular fluid.

Vertebrate smooth muscles (Manery & Bale, 1941; Wilkins and Cullen, 1933) contain a much higher proportion of sodium and chloride than striated muscle but this may be due, in part at least, to a higher proportion of extracellular fluid between the fibres. Bulbring & Born, quoted by Holman (1957), estimate that the extracellular fluid in the taenia coli muscle of the guinea-pig amounts to 36% of the total wet weight. A large number of analyses of invertebrate smooth muscle have been published, but as the analyses are rarely accompanied by measurements of the proportion of extracellular fluid it is difficult to draw any firm conclusions about the intracellular concentrations of ions. The analyses of the whole muscle usually show a relatively high concentration of sodium and chloride, sometimes amounting to as much as half the concentrations of the same ions in the blood (Singh, 1938; Krogh, 1939, p. 60; Steinbach, 1940), but it is not clear if this is accounted for by a high proportion of extracellular fluid or if the conditions of ionic equilibrium in invertebrate smooth muscle differ from those found in striated muscle.

To throw further light on this problem, measurements have been made of the concentrations of the chief ions—sodium, chloride and potassium—in the sarcoplasm of a variety of lamellibranch muscles. The lamellibranchs form a convenient

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source of substantial quantities of muscle uncontaminated by other tissues. Unfortunately, the small size of lamellibranch muscle fibres, usually about 8μ in diameter, makes it impracticable to analyse individual fibres; instead, analyses have been made of the whole muscle and the volume of contaminating blood has been estimated by inulin. The muscles which have been analysed are the anterior byssus retractor and the fast and slow portions of the adductor muscle of *Mytilus edulis*, and the fast and slow portions of the adductor muscle of *Pecten maximus*. The fast adductor of *Pecten* is striated and generally similar in physical properties to crab or frog muscle (Abbott & Lowy, 1956), and therefore provides a convenient link with better known muscles. The freshwater species *Anodonta cygnaea* has a very low concentration of ions in the blood and in the muscle, and analyses have been made of the two portions of the adductor muscles of this species. In order to widen still further the range of conditions, analyses have been made of *Mytilus* adductors and byssus retractors when the animals are adapted to 50% sea water, and of *Anodonta* adductors when the animals are adapted to 18% sea water.

Inorganic ions account for only a fraction of the osmotic pressure of the tissues of the marine species, so measurements have been made of the concentrations of phosphate compounds and free amino acids in order to define more closely the differences between the marine and freshwater species.

The fluxes of sodium through the muscle cells of the ventricles of *Mytilus* and *Anodonta* have also been measured and will be described in a later paper, but for convenience the results of the analyses of these two tissues are included here.

METHODS

The animals were kept in well-aerated tanks between 10° and 16° C. Both *Mytilus* and *Anodonta* gain sodium and lose potassium from the tissues when kept under laboratory conditions, so animals were used within 1 week of arrival, unless otherwise stated.

After excision and cleaning the muscles were blotted to remove surface fluid. The water content was taken as the loss of weight after drying overnight at 105° C.

Sodium and potassium were estimated by flame photometry using an 'EEL' flame photometer. Measurements were made at maximum dilution, 0.05–0.1 mm./l. The standard solutions used contained both sodium and potassium in similar concentrations to the samples analysed. When small quantities of muscle (5–50 mg.) were dry-ashed with concentrated sulphuric acid significant quantities of sodium were lost at 500° C. Complete solution without loss was obtained by evaporating to dryness on a water-bath with 0.1 ml. concentrated nitric acid and then dissolving in 5 or 10 ml. of water. Calcium was estimated by precipitating as oxalate and titrating with ceric sulphate (Robertson & Webb, 1939). Magnesium was precipitated as hydroxyquinolate and also titrated with ceric sulphate (Robertson & Webb, 1939). Chloride was estimated by the microdiffusion method of Conway (1947). Sulphate was estimated gravimetrically as barium sulphate after removing the protein with picric acid. Phosphate was estimated colorimetrically by the method of Fiske & SubbaRow (1925). Total free amino acids were estimated by

the method of Van Slyke, MacFadyen & Hamilton (1941). In this method the carbon dioxide evolved on decarboxylation with ninhydrin, indane-trione hydrate, is absorbed by barium hydroxide. Both the carboxyl groups in aspartic acid are decomposed by this method, so the relative amount of aspartic acid was estimated by paper chromatography. Two-dimensional chromatograms were run in phenol/water and butanol/acetic acid and water, and the spots developed with ninhydrin. After development the spots were extracted with 70% acetone and the relative intensities measured by a 'Spekker' absorptiometer. The method is not of high accuracy, but the aspartic acid is only about 5% of the total amino acids in *Mytilus*, so the correction is small. The method of Van Slyke *et al.* does not estimate taurine which was reported to occur in large quantities in *Mytilus* (Kelly, 1904). Taurine was removed from the muscle by repeated extraction with 70% alcohol and precipitated as barium sulphate, after boiling in bromine water for 4 hr. in alkaline solution. The method is not specific as other alcohol soluble compounds containing sulphur, such as isethionic acid, might also be oxidized to some extent. The chromatograms showed that amino acids containing sulphur were only present in small quantities. Hydroxycarboxylic acids were separated on a column of silica gel and estimated as described by Isherwood (1946), but the quantities found were very small.

The volume of extracellular fluid was estimated by injecting a suitable quantity of a 20% solution of inulin in sea water (or in dilute sea water) into a whole animal, and estimating the concentrations of inulin in the blood and in the muscle the following day. The inulin was estimated by the colorimetric method of Roe, Epstein & Goldstein (1949) using an 'EEL' absorptiometer. The blood was collected by hypodermic from the ventricle. The inulin was removed from the muscle by soaking 50–100 mg. portions of muscle, not exceeding 1 mm. in thickness, in a suitable saline for 5 hr., with frequent shaking. Extraction was 95% complete in an hour.

RESULTS

The adductor muscles of *Pecten* and *Mytilus* are large enough to allow several determinations of sodium, potassium and chloride to be made on one animal. Tables 1 and 2 show the results of a number of such analyses. Each figure for sodium and potassium is the mean of the analyses of three separate samples of muscle. Each chloride figure is the mean of two separate determinations. The water contents of the muscles are given in Table 3.

The ventricle of *Mytilus*, and to some extent the byssus retractor, are rather small for simultaneous determinations of sodium, potassium and chloride, so only the mean values and standard errors of a large number of separate determinations of these quantities are given (Table 4).

The sodium and chloride content of *Anodonta* muscle is very variable but much of the variation is clearly related to the variation in the blood composition. When expressed as a percentage of the concentration in the blood, the sodium and chloride results are more consistent (Table 5).

Table 1. *Sodium, potassium and chloride concentrations in the adductor muscle and the blood of Pecten maximus*
(mm./kg. total water.)

Animal	Fast adductor			Slow adductor		
	Na	K	Cl	Na	K	Cl
1	77	138	—	176	128	189
2	82	166	70.7	176	143	—
3	72	154	58.0	125	146	145
4	50	157	59.5	146	149	166
5	72	160	85.4	170	131	222
6	78	136	71.6	149	113	171
7	59.5	144	57.3	133	141	157
8	—	—	60.6	—	—	156
Mean (\pm standard error)	70 \pm 5	151 \pm 5	66.2 \pm 4	154 \pm 7	136 \pm 5	172 \pm 10
Blood	490	12.5 \pm 0.3	573			

Table 2. *Sodium, potassium and chloride concentrations in the adductor muscle of Mytilus edulis*
(mm./kg. total water.)

Animal	Fast adductor			Slow adductor		
	Na	K	Cl	Na	K	Cl
1	150	131	146	166	108	194
2	143	131	212	180	123	242
3	183	129	179	178	134	215
4	132	129	174	180	118	212
5	174	107	183	205	108	247
6	165	122	227	217	99	254
Mean (\pm standard error)	158 \pm 9	125 \pm 4	187 \pm 13	188 \pm 9	115 \pm 6	227 \pm 13

Table 3. *Water content of lamellibranch muscles*
(% total weight.)

	Fast adductor	Slow adductor	Ventricle	Byssus retractor
<i>Pecten</i>	76.3 \pm 0.6 (20)	74.6 \pm 0.8 (15)	—	—
<i>Mytilus</i>	75 \pm 0.6 (17)	75.6 \pm 0.6 (14)	80.8 \pm 0.6 (14)	78.4 \pm 0.5 (16)
<i>Mytilus</i> , 50 % sea water	78.1 \pm 0.6 (8)	78.9 \pm 0.3 (8)	—	80.7 \pm 0.4 (8)
<i>Anodonta</i> , fresh water	83.7 \pm 0.6 (6)	85.1 \pm 0.4 (6)	87.8 \pm 0.8 (8)	—
<i>Anodonta</i> , 18 % sea water	75.8 \pm 1.0 (4)	79.2 \pm 1.0 (4)	—	—

Mean \pm standard error (no. of observations).

The muscles of the marine species show very wide but systematic variations in their sodium, potassium and chloride content. The muscles may be arranged in a series on the basis of their inorganic content: *Pecten* fast adductor, *Pecten* slow adductor, *Mytilus* fast adductor, *Mytilus* ventricle, *Mytilus* slow adductor, *Mytilus* byssus retractor. In this series the sodium and chloride contents of the muscles

Table 4. Sodium, potassium and chloride content of the anterior retractor byssus, the ventricle and the blood of *Mytilus edulis*

(mm./kg. total water.)

	Na	K	Cl
Retractor byssus	208 ± 10 (18)	107 ± 3 (18)	281 ± 4 (12)
Ventricle	181 ± 11 (16)	120 ± 3 (14)	190 ± 10 (12)
Blood	490	12.5 ± 0.2 (12)	573

Mean ± standard error (no. of observations).

Table 5. Concentrations of sodium, potassium and chloride in the adductor muscles and the ventricle of *Anodonta cygnaea*

(mm./kg. total water. The sodium and chloride are also expressed as % of the blood concentration.)

	Na		K	Cl	
	mm./kg. total water	% of blood concn.	mm./kg. total water	mm./kg. total water	% of blood concn.
Fast adductor	6.6	45 ± 2.9 (6)	18.4 ± 1.0 (8)	3.86	36.7 ± 4.3 (6)
Slow adductor	9.2	63 ± 4.0 (6)	12.4 ± 1.0 (8)	6.82	64.2 ± 3.9 (6)
Ventricle	9.5	65 ± 1.7 (8)	10.5 ± 0.6 (12)		
Blood	14.7 ± 1.3	(8)	0.45 ± 0.014 (14)	10.7 ± 1.0	(6)

Mean ± standard error (no. of observations).

are steadily increasing and the potassium contents of the muscles are steadily falling. It is also the order of increasing time of stress relaxation (or, more generally, decreasing speed of muscle) where this is known (Abbott & Lowy, 1956, 1957). The fastest muscle, the fast portion of *Pecten* adductor, is similar to *Carcinus* striated muscle in that it contains little sodium or chloride, whereas the slowest muscle, the byssus retractor, contains almost half as much sodium and chloride as the blood.

The concentrations of ions found in the *Anodonta* muscles are necessarily much lower than in the marine species because the blood has a total osmolar concentration of only 4–5 % of sea water, but otherwise the picture is similar. The slower muscle contains the greater quantities of sodium and chloride, amounting in this case to more than half of the blood concentration.

The intracellular concentrations of sodium and chloride

The whole muscle contains considerable quantities of extracellular fluid which must be allowed for when calculating the intracellular concentrations of the ions. The extracellular fluid has been assumed to be identical with the space accessible to inulin (Table 6). The sodium, potassium and chloride contents of the blood of *Anodonta* are given in Table 5. The sodium and chloride content of the blood of *Pecten* and of *Mytilus* is assumed to be the same as that of the sea water in which they had been kept before analysis. The potassium content of the blood of *Pecten*

Table 6. *Inulin space in lamellibranch muscle expressed as % of total water content*

	Fast adductor	Slow adductor	Ventricle	Byssus retractor
<i>Pecten</i>	6.0 ± 0.26 (19)	24 ± 0.6 (18)	—	—
<i>Mytilus</i>	19.3 ± 0.9 (16)	24.5 ± 1.3 (14)	26.0 ± 3.4 (4)	29.5 ± 1.2 (12)
<i>Mytilus</i> , 50% sea water	14.1 ± 0.9 (7)	21.3 ± 2.0 (7)	—	25.3 ± 1.1 (6)
<i>Anodonta</i> , fresh water	14.4 ± 1.2 (13)	27.4 ± 1.4 (12)	30.5 ± 2.5 (5)	—
<i>Anodonta</i> , 18% sea water	41.5 ± 1.6 (11)	46.4 ± 2.9 (7)	—	—

Mean ± standard error (no. of observations)

and *Mytilus* is rather higher than that of sea water, and was measured at the same time as the potassium content of the muscle (Tables 1 and 4). The mean sodium content of the blood of *Anodonta* is rather lower than a previously published figure by the author (Potts, 1954). This is probably because Birmingham tap water has a much lower sodium content than Cambridge tap water.

Table 7. *Concentrations of sodium, potassium and chloride in some lamellibranch muscles*

	(1) Whole muscle (mm./kg. total water)			(2) Blood space (mm./kg. water)			(3) Sarcoplasm (mm./kg. fibre water)		
	Na	K	Cl	Na	K	Cl	Na	K	Cl
<i>Pecten</i>									
Fast adductor	70 ± 5	151 ± 5	66.2 ± 4	29.4 ± 1	1	34.4 ± 1	43 ± 5	160 ± 5	34 ± 5
Slow adductor	154 ± 7	136 ± 5	172 ± 10	88 ± 3	2	104 ± 3	81 ± 10	163 ± 6	82 ± 5
<i>Mytilus</i>									
Fast adductor	158 ± 9	125 ± 4	187 ± 13	94 ± 4	2	111 ± 5	79 ± 12	152 ± 5	94 ± 4
Slow adductor	188 ± 9	115 ± 6	227 ± 13	120 ± 6	3	140 ± 7	90 ± 15	148 ± 8	115 ± 5
Byssus retractor	212 ± 9	112 ± 3	276 ± 4	145 ± 6	4	169 ± 7	95 ± 16	153 ± 4	152 ± 5
Ventricle	181 ± 10	120 ± 4	190 ± 9	127 ± 17	3	149 ± 19	73 ± 26	158 ± 5	56 ± 4
<i>Mytilus</i> , 50% sea water									
Fast adductor	58.0 ± 6	91.4 ± 5	60.0 ± 4	34.5 ± 2	0.7	40.5 ± 3	27 ± 7	106 ± 6	23 ± 5
Slow adductor	82.5 ± 5	76.6 ± 6	74.6 ± 4	52.2 ± 5	1.1	61.2 ± 6	38 ± 9	96 ± 8	17 ± 5
Byssus retractor	79.8 ± 6	85.5 ± 4	92.5 ± 6	62.0 ± 3	1.4	72.6 ± 3	24 ± 9	112 ± 5	27 ± 5
<i>Anodonta</i> , fresh water									
Fast adductor	6.6 ± 0.4	18.4 ± 1.0	3.9 ± 0.5	2.1 ± 0.3	0.06	1.54 ± 0.23	5.3 ± 0.6	21.3 ± 1.2	2.4 ± 0.4
Slow adductor	9.2 ± 0.6	12.4 ± 1.0	6.8 ± 0.4	4.0 ± 0.3	0.12	2.9 ± 0.3	7.2 ± 0.9	16.9 ± 1.4	5.4 ± 0.4
Ventricle	9.5 ± 0.2	10.5 ± 0.6	—	4.5 ± 0.4	0.14	—	7.2 ± 0.7	14.9 ± 0.9	—
<i>Anodonta</i> , 18% sea water									
Fast adductor	64.6 ± 2.1	26.1 ± 0.4	48.1 ± 2.0	35 ± 1.8	0.3	31 ± 1.8	50 ± 4.8	44 ± 2.0	29 ± 4.8
Slow adductor	65.8 ± 3.1	20.0 ± 0.4	59.2 ± 1.7	39 ± 2.7	0.3	35 ± 2.6	50 ± 7.6	45 ± 2.9	55 ± 4.8

Mean ± standard error.

The intracellular concentrations of sodium, potassium and chloride (Table 7, column 3) are calculated from the analyses of the whole muscles (Table 7, column 1) and from the quantities of ions in the inulin spaces of the muscle (Table 7, column 2). The results are expressed in mm./kg. fibre water. The intracellular concentrations of sodium and chloride are calculated from the small difference between two large

and variable quantities, and so are peculiarly subject to error. However, the results are fairly consistent, and show that as the speed of the muscle increases, the intracellular sodium and chloride fall and, to a lesser extent, potassium rises. In the fast adductor of *Pecten* the intracellular concentrations of sodium and chloride are less than one-tenth of the concentrations of the same ions in the blood, and in this respect the muscle is similar to vertebrate striated muscle. In the slow adductor muscles, especially the slow adductor of *Anodonta*, the relative intracellular concentrations of chloride are very much higher.

Other intracellular constituents

The total osmolar concentration inside the muscle fibres of *Anodonta* is only about one-twentieth of that of the marine species. Fuller analyses have been made of the ventral adductors of *Mytilus* and *Anodonta* to define more clearly the differences between the marine and freshwater species.

The calcium, magnesium and sulphate concentrations in *Mytilus* (Table 8) are

Table 8. Concentrations of calcium, magnesium and sulphate in the fast portions of the adductor muscles of *Mytilus* and *Anodonta*

(Each figure is the mean of four determinations.)

	Blood (mm./kg. water)	Whole muscle (mm./kg. total water)	Sarcoplasm (mm./kg. fibre water)
Magnesium			
<i>Mytilus</i>	56	38	34
<i>Anodonta</i>	0.2	3.9	4.5
Calcium			
<i>Mytilus</i>	12.6	8.55	7.3
<i>Anodonta</i>	8.4	11.5	12.0
Sulphate			
<i>Mytilus</i>	30.7	13.0	8.8

broadly similar to those found in the muscles of other marine animals. Shaw (1955) reported a calcium concentration of 5.4 mm./kg. fibre water in the muscles of *Carcinus maenas*. The magnesium concentration in the ventral adductor of *Mytilus* is about twice as great as in *Carcinus*, 34 mm./kg. fibre water compared with 16 mm./kg. fibre water, but the concentration of magnesium in the blood is correspondingly higher, 56 mm./kg. water compared with 21.2 mm./kg. water. The concentration of magnesium in *Anodonta* blood, 0.02 mm./kg. water, is exceedingly low, but the muscle still contains 5.2 mm./kg. fibre water. Florkin & Duchâteau (1950) reported a magnesium concentration of 7.1 mm./kg. fibre water; the fibre water was calculated on the assumption that all the chloride was extracellular and the portion of the adductor analysed was not specified.

Phosphagen phosphate, inorganic phosphate and adenosine triphosphate have been measured in both *Mytilus* and *Anodonta* (Table 9). The highest concentration of phosphate in whole muscle is found in the fast portion of *Mytilus* adductor. Some of the difference between the two portions of the adductor is due to the

greater proportion of blood in the slow muscle, but the difference is not abolished even when allowance is made for this extracellular fluid. The freshwater animal contains about half the phosphate concentration of the marine animal in spite of its very much lower blood concentration. The phosphate compounds were conserved more effectively than any other measured variable when the Unionidae became adapted to fresh water.

Table 9. *Phosphate content of Mytilus and Anodonta muscle*

(Each figure is the mean of four determinations.)

	Fast adductor	Slow adductor	Byssus retractor
Whole muscle (mm./kg. total water)			
<i>Mytilus</i>			
Arginine phosphate	13.4	7.8	13.5
Adenosinetriphosphate	4.9	4.9	4.7
Inorganic phosphate	13.3	12.8	6.3
<i>Anodonta</i>			
Arginine phosphate	8.4	6.3	—
Adenosinetriphosphate	1.7	0.95	—
Inorganic phosphate	6.9	3.9	—
Sarcoplasm (mm./kg. fibre water)			
<i>Mytilus</i>			
Total phosphate	39.0	33.6	34.6
<i>Anodonta</i>			
Total phosphate	19.8	14.0	—

The concentrations of free amino acids in *Mytilus* and *Anodonta* were estimated by measuring the amount of carbon dioxide evolved on decarboxylation with ninhydrin. Aspartic acid, but not glutamic acid, decomposes to give 2 mmoles of carbon dioxide for 1 mmole of acid. Taurine, a sulphonic acid, is not estimated by this method. Taurine and aspartic acid were estimated separately by other means, and the total free amino acids calculated by adding taurine to the amount estimated by decarboxylation and subtracting the amount of aspartic acid (Table 10). Kelly (1904) estimated that taurine made up 5% of the dry weight of *Mytilus* muscle, corresponding to 100 mm./kg. water in the whole muscle. The chromatograms show that *Mytilus* muscle contains considerable quantities of glycine, alanine, glutamic acid and arginine and some histidine, tyrosine and threonine. *Anodonta* muscle contains glycine, arginine and glutamic acid. The concentration of amino acids in *Mytilus* blood is only 2.5 mm./kg. water, and is even lower, 0.5 mm./kg. water, in *Anodonta*. Duchâteau, Sarlet, Camien & Florkin (1952) estimated the quantities of free amino acids, excluding taurine, in *Mytilus* and *Anodonta* by bio-assay. They found that *Mytilus* muscle contained 166.2 mm./kg. total water and *Anodonta* muscle contained 9.5 mm./kg. total water; the part of the adductor assayed was not defined.

Hydroxycarboxylic acids occur in very low concentrations in lamellibranch muscle. Only 2.4 mm./kg. total water were recovered from *Mytilus* muscle.

Table 10. *Free amino acids in Mytilus and Anodonta*

(Each figure is the mean of six determinations.)

	mm./kg. total water				mm./kg. fibre water. Total sarcoplasm
	Carboxylic acid*	Taurine	Aspartic acid	Total whole muscle	
<i>Mytilus</i>					
Ventral adductor	155 ± 3	91 ± 4	8	238 ± 5	295 ± 6
Byssus retractor	126 ± 4	75 ± 3	8	193 ± 5	273 ± 6
Blood	2.5 ± 1	—	—	—	—
<i>Mytilus</i> , 50 % sea water					
Ventral adductor	93.5 ± 4	66 ± 4	6	153.5 ± 5	183 ± 6
Byssus retractor	73.4 ± 4	57 ± 3	6	124.4 ± 5	166 ± 6
Blood	5.4 ± 2	—	—	—	—
<i>Anodonta</i>					
Ventral adductor	10.5 ± 2	0	1	9.5 ± 2	11.0 ± 2
Blood	0.47 ± 0.4	—	—	—	—
<i>Anodonta</i> , 18 % sea water					
Ventral adductor	30 ± 4	0	2	28 ± 4	48 ± 7

* By the method of Van Slyke, MacFadyen & Hamilton (1941).

The total osmotic pressure of the identified constituents

The sea water in which *Mytilus* had been living contained 573 mm./kg. water of chloride. This corresponds to 19.6‰ chlorinity, a freezing-point depression of 1.89° C. or an osmolar concentration of just over 1. The identified constituents of *Mytilus* muscle amount to less than 700 mm./kg. fibre water (Table 11). The totals given in Table 11 are themselves too high because in the resting muscle much of the arginine and phosphate, which are included separately in Table 11, are combined. On the other hand, no account is taken in Table 11 of calcium, magnesium or sulphate which may contribute to some extent to the osmotic pressure.

Table 11. *Major constituents of the osmotic pressure of Mytilus and Anodonta muscle*

(mm./kg. fibre water.)

	<i>Mytilus</i>		<i>Anodonta</i> . Fast adductor
	Fast adductor	Byssus retractor	
Na	79	95	5.3
K	152	134	21.3
Cl	94	152	2.4
PO ₄	39	35	19.8
Amino acids	289	273	11.0
	653	689	59.8

The assayed osmotic constituents of *Anodonta* muscle amount to a total of 60 mm./kg. fibre water, but the muscle contains at least 8.4 and possibly as much as 10 mm. arginine phosphate/kg. fibre water in the resting state. The mean freezing-point depression of the blood of a large series of *Anodonta* amounted to

0.078° C. or 42 mM./kg. water (Potts, 1954). Some of the inorganic phosphate recorded in Table 9 may be in compounds not exerting an equivalent osmotic pressure. Calcium and manesium in *Anodonta* muscle (Table 8) must be largely combined with organic molecules.

CHANGES IN THE COMPOSITION OF THE MUSCLE IN RESPONSE TO A FALL IN THE CONCENTRATION OF THE BLOOD

Mytilus occurs in estuaries and can tolerate considerable changes in the composition of the blood. The sodium, potassium and chloride contents of muscles from *Mytilus* which had been living for at least 2 weeks in 50% sea water are given in Table 12.

Table 12. *Sodium, potassium and chloride content of some tissues of Mytilus living in 50% sea water*
(mM./kg. total water.)

	Na	K	Cl
Sea water	245	5.2	287
Blood	245	7.4 ± 0.26 (12)	286
Fast adductor	58.0 ± 6 (14)	91.4 ± 5 (14)	60.0 ± 4 (10)
Slow adductor	82.5 ± 5 (12)	76.6 ± 6 (12)	74.6 ± 4 (10)
Byssus retractor	79.8 ± 6 (12)	85.5 ± 4 (12)	92.5 ± 6 (10)

Mean ± standard error (no. of observations).

The percentage dry weight of the muscles is less than that of muscles from normal sea water (Table 3), and the inulin spaces have also been reduced (Table 6), so the individual muscle fibres must be osmotically swollen. From the figures in Tables 3 and 6 it follows that, when allowance is made for the solids in the blood, 100 g. of solids are associated with 247 g. of water in the fibres of the fast adductor of *Mytilus* from normal sea water, but are associated with 311 g. of water in animals from 50% sea water. Similarly, in the byssus retractor 100 g. of solids are associated with 266 g. of water in normal sea water and 319 g. in 50% sea water. In each case the distension is much less than the doubling of water content which would occur if the muscles behaved as simple osmometers. The fall in the potassium concentration of the muscles is a little greater than that caused by the osmotic uptake of water alone, but the fall in the sodium and chloride concentrations is very much greater. In the *Mytilus* muscles in normal sea water the average intracellular concentrations of sodium and chloride are 88 and 87 mM./kg. water respectively (Table 7). In 50% sea water these concentrations have fallen to 30 and 22 mM./kg. water.

The amino-acid content of the muscle also falls by an amount greater than that due to water uptake alone. In the normal ventral adductor the intracellular concentration of free amino acids is 295 ± 6 mM./kg. water (Table 10). In 50% sea water this falls to 183 ± 6 mM./kg. water. The uptake of water should have reduced the concentration to 234 mM./kg. water; the remainder of the reduction must be caused by the excretion, polymerization or metabolism of the amino acids. In the byssus retractor the intracellular concentration of free amino acids is 273 ± 6 mM./kg. water when the animals are in sea water, and only 166 ± 6 mM./kg. water when the

animals are in 50% sea water (Table 10). The osmotic swelling of the muscle fibres alone would reduce the concentration to 228 mM./kg. water. The concentration of free amino acids in the blood has risen to 5.4 mM./kg. water when the animal is in 50% sea water from 2.5 mM./kg. water in normal sea water. Some of the amino acids in the blood are probably derived from the muscles.

Analyses of *Anodonta* which had been living for 10 days in 18% sea water are recorded in Tables 3, 6, 7, 10 and 13. The percentage dry weights of the muscles

Table 13. *Sodium, potassium and chloride content of some tissues from Anodonta living in 18% sea water*
(mM./kg. total water.)

	Na	K	Cl
Sea water	87.0	1.92	102
Blood	84.0 ± 3 (6)	0.72 ± 0.1 (8)	75 ± 3 (6)
Fast adductor	64.6 ± 2.1 (6)	26.1 ± 0.4 (10)	48.1 ± 2.0 (6)
Slow adductor	65.8 ± 3.1 (6)	20.0 ± 0.4 (10)	59.2 ± 1.7 (6)
Ventricle	63.0 ± 4.2 (6)	27.7 ± 0.9 (10)	—

Mean ± standard error (no. of observations).

and the inulin spaces have increased in 18% sea water. In an *Anodonta* from tap water 100 g of dry matter in the ventral adductor are associated with 440 g. of intracellular water. In 18% sea water this has fallen to 187 g. of intracellular water. In the dorsal adductor the figures are 415 and 204 g. respectively. Exosmosis has removed over half the fibre water. Correspondingly, the intracellular concentrations of potassium have also doubled in 18% sea water (Table 7), but the intracellular concentrations of sodium and chloride have increased eight- or tenfold.

In both *Anodonta* and *Mytilus* adaptation to a changed osmolar concentration is brought about partly by water movement into or out of the muscle fibres, and partly by the increase or decrease of the total content of sodium, chloride and free amino acids in the muscles.

DISCUSSION

Sodium, potassium and chloride equilibria

In many of the muscles analysed the distribution of potassium and chloride across the fibre membrane appears to differ significantly from a Donnan equilibrium. For example, in the byssus retractor muscle of *Mytilus* from normal sea water the potassium concentration ratio between the inside and the outside of the fibres is 12.2 ± 0.4 , while the chloride is 3.9 ± 0.3 . Similarly, in *Anodonta* slow adductor the potassium ratio is 38 ± 3.0 , while the chloride ratio is only 2.0 ± 0.35 (Table 14). In vertebrate striated muscle, in some invertebrate striated muscle and in many vertebrate and invertebrate nerves it is well established that the distribution of potassium and chloride approximates to a Donnan equilibrium (Boyle & Conway, 1941; Hodgkin, 1951; Shaw, 1955). The last two columns of figures in Table 14 therefore require careful consideration. The fast adductor of *Pecten*, the only

Table 14. *Concentration ratios of sodium, potassium and chloride ions inside and outside some lamellibranch muscle fibres*

	$\frac{Na_o}{Na_i}$	$\frac{K_i}{K_o}$	$\frac{Cl_o}{Cl_i}$
<i>Pecten</i>			
Fast adductor	11.4 ± 1.2	12.8 ± 0.5	16.8 ± 2.0
Slow adductor	6.0 ± 0.8	13.0 ± 0.6	6.9 ± 1.1
<i>Mytilus</i>			
Fast adductor	6.2 ± 1.0	12.2 ± 0.6	6.1 ± 1.1
Slow adductor	5.5 ± 0.9	11.8 ± 0.6	5.0 ± 0.9
Byssus retractor	5.2 ± 0.9	12.2 ± 0.4	3.9 ± 0.3
Ventricle	6.7 ± 2.5	12.6 ± 0.4	10.2 ± 7.2
<i>Mytilus</i> , 50 % sea water			
Fast adductor	9.1 ± 2.5	14.3 ± 0.8	12.0 ± 1.3
Slow adductor	6.4 ± 1.6	13.0 ± 1.2	16.8 ± 12.8
Byssus retractor	10.2 ± 4.4	15.1 ± 10.7	10.2 ± 3.8
<i>Anodonta</i>			
Fast adductor	2.8 ± 0.36	47 ± 3	4.5 ± 1.3
Slow adductor	2.0 ± 0.31	38 ± 3	2.0 ± 0.35
<i>Anodonta</i> , 18 % sea water			
Fast adductor	1.67 ± 0.18	61 ± 2.7	2.58 ± 0.4
Slow adductor	1.67 ± 0.25	63 ± 4.5	1.36 ± 1.5

muscle in the table comparable to vertebrate striated muscle or to *Carcinus* muscle in physical properties, has potassium and chloride concentration ratios close to a Donnan equilibrium. In the remaining muscles it is generally the slower muscles which differ most from the equilibrium conditions. Either the principles governing ionic equilibrium in lamellibranch smooth muscles differ from those in striated muscle, or some important factor has been neglected in calculating the results in Table 7.

In every case the apparent high concentration of chloride inside the fibres is associated with a high concentration of sodium. If the inulin space is not identical with the extracellular fluid, but is for some reason somewhat smaller, then the calculated concentrations of sodium and chloride inside the fibres will be too high. For example, if it is assumed that all the sodium in the byssus retractor muscle of *Mytilus* is extracellular, then the extracellular space would amount to 43 % of the total water content, the intracellular chloride concentration would be reduced to 46 mM./kg. water and the intracellular potassium would be increased to 191 mM./kg. water which would be close to the conditions of a Donnan equilibrium. This could be the case if the muscle contains a third phase, neither blood nor sarcoplasm, inaccessible to inulin but containing large quantities of sodium and chloride. Such a third phase could be formed either by connective tissue between the fibres or by a specialized region of the fibres such as the sarcolemma. In the vertebrates connective tissue contains large quantities of sodium and chloride (Manery & Hastings, 1939). Olson (1938) described connective tissue between the fibres of *Thyone* muscle, which he correlated with the high chloride content of the muscle, but an histological examination of *Mytilus* byssus retractor by the present author,

using the same technique as Olsen (that is, fixing with Bouin and staining with Mallory's triple stain) revealed only negligible amounts of connective tissue.

Carey & Conway (1954) have described experiments on frog sartorii from which they conclude that most of the non-extracellular sodium is in the sarcolemma and only a small quantity is in the sarcoplasm. In order to account for the discrepancy between the observed and the equilibrium concentrations in *Mytilus byssus* retractor the sarcolemma would have to contain the very large quantity of 100 mM. Cl/kg. muscle. It is difficult to reconcile the potassium and chloride content of lamellibranch smooth muscle with Conway's model of a simple Donnan equilibrium.

In a recent review Robertson (1957) has discussed the distribution of potassium and chloride ions in the muscles of a number of invertebrates. On the basis of his own analysis of *Nephrops*, Shaw's analysis of *Carcinus* (1955) and Krogh's analysis of *Mytilus* and *Eriocheir* (1939) he concludes that only in *Carcinus* does the distribution approximate to a Donnan equilibrium. In the remaining animals the chloride concentration inside the fibres exceeds the equilibrium concentration.

Hodgkin (1958) has discussed ionic equilibria in systems in which the permeability to sodium is low compared with the permeability to potassium but not completely negligible, and in which a neutral pump operates absorbing one ion of potassium for each ion of sodium extruded. In these circumstances the equilibrium potential E is given by a modified Nernst equation

$$E = \frac{RT}{F} \ln \frac{[K_o] + b[Na_i]}{[K_i] + b[Na_o]},$$

where R is the universal gas constant, T is the absolute temperature, F is the faraday, K_o and Na_o ; K_i and Na_i the concentrations of potassium and sodium outside and inside the system respectively and b is the permeability to sodium relative to the permeability to potassium. If the chloride ion is under no restraint other than the resting potential then

$$E = \frac{RT}{F} \ln \frac{[Cl_i]}{[Cl_o]},$$

and

$$\frac{[K_o] + b[Na_o]}{[K_i] + b[Na_i]} = \frac{[Cl_i]}{[Cl_o]}.$$

Values of b have been calculated for the analysed muscles from the data in Tables 1, 4, 5, 7, 12 and 13 on the assumption that these conditions apply in lamellibranch muscle (Table 15). b increases with decreasing speed of muscle. It does not differ significantly from zero in the fast adductor of *Pecten* but rises to 0.059 ± 0.007 in the byssus retractor of *Mytilus* and to 0.71 ± 0.14 in the slow adductor of *Anodonta*. In *Mytilus* from 50% sea water the standard errors have accumulated so much that the results are hardly significant, but the values of b calculated for *Anodonta* from 18% sea water are very close to those calculated for *Anodonta* from fresh water. This is not the only possible system consistent with the experimental results but as the potassium and chloride ions are not in a Donnan equilibrium any alternative explanation must involve the active transport of either potassium or chloride or both in addition to the transport of sodium.

Table 15. $b = \frac{\text{permeability to potassium}}{\text{permeability to sodium}}$ in lamellibranch muscle, calculated from Tables 9 and 10

Animal	Tissue	b
<i>Pecten</i> , 100 % sea water	Fast adductor	-0.006 ± 0.003
	Slow adductor	0.023 ± 0.008
<i>Mytilus</i> , 100 % sea water	Fast adductor	0.026 ± 0.009
	Slow adductor	0.036 ± 0.011
	Byssus retractor	0.059 ± 0.007
	Ventricle	0.006 ± 0.016
<i>Mytilus</i> , 50 % sea water	Fast adductor	0.006 ± 0.010
	Slow adductor	-0.007 ± 0.012
	Byssus retractor	0.013 ± 0.014
<i>Anodonta</i> , fresh water	Fast adductor	0.32 ± 0.09
	Slow adductor	0.71 ± 0.14
<i>Anodonta</i> , 18 % sea water	Fast adductor	0.26 ± 0.04
	Slow adductor	0.69 ± 0.09

SUMMARY

1. Measurements have been made of the inorganic ion and free amino acid content of a number of lamellibranch muscles. The volumes of extracellular fluid in the muscles have also been determined so that the intracellular concentrations can be calculated.

2. The fast portion of the adductor muscle of *Pecten* contains about 160 mM. K/kg. fibre water and only 43 mM. Na/kg. fibre water and 34 mM. Cl/kg. fibre water. The potassium and chloride are approximately in a Donnan equilibrium with the potassium and chloride in the blood.

3. In the slow portion of the adductor muscle of *Pecten* and in the two parts of the adductor and in the byssus retractor of *Mytilus*, the concentrations of potassium in the fibres are from 150 to 160 mM./kg fibre water, of sodium 73 to 95 mM./kg. fibre water and chloride 94 to 152 mM./kg. fibre water. The potassium and chloride in the fibres are not in a Donnan equilibrium with the potassium and chloride in the blood.

4. The fast and slow fibres of the adductor muscles of *Anodonta* contain 21 and 17 mM./kg. fibre water of potassium respectively, 5.3 and 7.2 mM./kg. fibre water of sodium, and 2.4 and 5.4 mM./kg. fibre water of chloride. The potassium and chloride in the fibres is not in a Donnan equilibrium with the potassium and chloride in the blood.

5. The fast fibres of *Mytilus* adductor contain 295 mM./kg. fibre water of free amino acids and 39 mM./kg. fibre water of acid-soluble phosphate compounds. The fast fibres of *Anodonta* adductor contain only 11 mM./kg. fibre water of amino acids and 19.8 mM./kg. fibre water of phosphate compounds.

6. *Mytilus* muscles fibres adapt to a reduced blood concentration, partly by an increase in water content and partly by a reduction in the sodium, chloride and free amino acid content.

7. *Anodonta* muscle fibres adapt to an increased blood concentration, partly by a reduction in the water content and partly by an increased sodium and chloride content.

8. The significance of these results is discussed. It is concluded that the ionic contents of the lamellibranch smooth muscles are consistent with equilibria systems in which the permeability to sodium is significant compared with the permeability to potassium and in which both a sodium and a potassium pump operate.

I am grateful to the Director of the Marine Biological Association Laboratory, Plymouth, for hospitality and facilities during the course of part of this work.

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SOME EXPERIMENTS ON FORM PERCEPTION IN THE NYMPHS OF THE DESERT LOCUST, *SCHISTOCERCA GREGARIA* FORSKÅL.

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(Received 28 March 1958)

INTRODUCTION

During an investigation into the importance of the visual sense in the behaviour of the desert locust it was found that individuals were attracted towards conspicuous objects in their environment and, if given a choice, showed a preference for certain shapes of object. The present paper is an account of the experiments performed to study this response in more detail.

Although these experiments are by no means exhaustive, their results indicate a few of the figural properties which are important to this insect. Spontaneous attraction towards objects in the visual environment and discrimination between the shapes of these objects has been shown in the case of several insects. It has been studied in bees (Hertz, 1929-37; Wolf & Zerrahn-Wolf, 1934, etc.; see References), in butterflies (Ilse, 1932), in caterpillars (Hundertmark, 1937*a*), and in grasshoppers (Williams, 1954). In this paper a general comparison is made between this previous work and the present results, and suggestions are advanced as to the possible basis of form discrimination in the desert locust.

METHOD AND MATERIAL

The principle of the experiments was to present an insect with two types of object in the visual field and to study its relative attraction to them.

The apparatus consisted of a circular arena 2 ft. in diameter with a wall 10 in. high. The wall was of white card and the floor was covered with white paper which was renewed from time to time. The objects were pieces of black card fastened on to the wall of the arena. Eight objects were used in each test, four of one shape and four of another. These shapes were arranged at equal intervals round the wall, the different shapes alternating with each other. In this way it was hoped to ensure that an insect placed in the centre of the arena had an equal chance of seeing both types of object. Light was supplied from a 100 W. opaque bulb hung 2 ft. above the centre of the arena and surrounded by a white shade. The experiments were performed in a darkened constant temperature room maintained at 28° C.

The animals used were nymphs of the phase *gregaria* of the desert locust *Schistocerca gregaria* Forskål. In most cases fifth-instar nymphs were used, but

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when unavailable they were replaced by fourths. These details are given in the table of results. There appeared to be no difference in behaviour between the two instars as can be seen from the experiments in which both were used (Exps. 2 and 9).

The animals were fed 1 hr. before testing.

OBSERVATIONS

An insect was placed gently in the centre of the arena and observed. It was found that it usually remained stationary for a short period (about a quarter of a minute). It then swayed the front part of the body slowly from side to side several times before starting to walk. This swaying motion is called 'peering' (Kennedy, 1945). After peering the insect set off and continued to walk until it reached the wall of the arena. During this journey it might pause from time to time, peer and then continue in the same direction as before or change to a new direction. In most cases an insect would go to one of the black objects present.

A run was terminated when the insect first touched the arena wall and this point was noted, taking the centre of the insect's head as the reference. A hit was scored if this point was on the black object or within 0.25 in. of the edge. This latter criterion was seldom needed because the orientation to the object edge was extremely accurate. Misses occurred when an insect touched the arena wall at a white part. (In a few experiments special criteria had to be used because of the nature of the objects; details of these are given in the appropriate parts of the text.) Several runs were made with the same animal (see Table 1). After every three runs or so the arena was rotated with respect to the floor. The paper floor was replaced when it had become marked (e.g. by excreta or regurgitated gut content). These precautions were to ensure that the choice was not biased by a tendency on the part of the insect to walk in any particular direction with respect to stable features of the environment, e.g. the bulb, or to follow any sort of scent track. Neither of these changes produced any disturbance of behaviour.

When an insect was placed in the arena care was taken not to point it directly at one object.

RESULTS

The results are presented in Table 1 and summarized pictorially in Fig. 1. On the null hypothesis that there is no attraction to the objects as compared to the white background the insects should pay equal visits to all parts of the wall. Thus the proportion of hits to misses should be in the same ratio as the respective lengths of circumference occupied by the figures and the white spaces. In fact it can be calculated that the actual ratio of hits to misses obtained differs highly significantly from this expectation. In all cases the number of misses is less than half the total number of hits. In comparing the relative attractiveness of each of a pair of figures therefore the misses are ignored and the distribution of hits is compared with a chance 50:50 distribution by χ^2 . The values of P as calculated by this method are given in the table.

Table 1. *The results obtained in spontaneous choice experiments to study form discrimination in the nymphs of the desert locust*
Schistocerca gregaria

(The table includes a verbal description of the figures used and these are also shown in Fig. 1)

Figures		Results		P χ^2	Animals	
		Hits	Misses		Instar	No.
Rectangles of equal breadth; different height, area and perimeter	Rectangle 10 in. \times 2 in. Rectangle 5 in. \times 2 in.	34 6	0	<0.01	5	40 each, 5 times
Rectangles of equal height; different breadth, area and perimeter	Rectangle 10 in. \times 2 in. Rectangle 10 in. \times 4 in.	66 22	12	<0.01	5	10 each, 10 times
Rectangles of equal height; different breadth, area and perimeter	Rectangle 10 in. \times 2 in. Rectangle 10 in. \times 4 in.	28 9	13	<0.01	4	10 each, 5 times
Same as 2 but with broad rectangle subdivided by extra vertical white stripe	Rectangle 10 in. \times 2 in. Rectangle 10 in. \times 4 in. with vertical white stripe 0.25 in. broad	30 41	29	0.1-0.2	4	20 each, 5 times
Control for 3a, same as 2	Rectangle 10 in. \times 2 in. Rectangle 10 in. \times 4 in.	26 16	8	0.1-0.2	4	10 each, 5 times
Rectangles of equal height; different area, breadth and perimeter	Rectangle 5 in. \times 2 in. Rectangle 5 in. \times 0.25 in.	50 29	21	0.01-0.02	4	20 each, 5 times
Rectangles of equal area; different height and perimeter	Rectangle 6 in. \times 1 in. Rectangle 3 in. \times 2 in.	72 9	19	<0.01	5	10 each, 10 times
Rectangles of equal area and equal perimeter; different height	Rectangle 2 in. \times 1 in. vertical Rectangle 2 in. \times 1 in. horizontal	43 17	30	<0.01	4	10 each, 10 times
Equal panels of black and white	Black 10 in. \times 9 in. White 10 in. \times 9 in. Edges	4 4 22	0	<0.01	5	30 each, once
Straight sided figures; one vertical, one at 45°	Rectangle 6 in. \times 1 in. vertical Stripe 1 in. broad oblique	40 17	3	<0.01	5	12 each, 5 times
Figures with long axes vertical, equal height; one straight, one wavy	Rectangle 8 in. \times 1 in. Wavy stripe 8 in. tall, 1 in. broad	75 20	5	<0.01	5	20 each, 5 times
Same as 9a	Rectangle Wavy stripe	46 18	11	<0.01	4	15 each, 5 times
Figures with long axes vertical, equal height; one straight, one serrated and of different area	Rectangle 8 in. \times 1 in. Vertical figure 8 in. tall 1 in. max. breadth, 16 serrations/side	114 65	21	<0.01	5	40 each, 5 times
Figures with long axes vertical, equal height, equal area; one straight, one serrated	Rectangle 8 in. \times 2 in. Vertical figure 8 in. tall, 2.5 in. max. breadth, 8 serrations/side	30 15	10	0.02-0.05	5	11 each, 5 times
Tall serrated figure, short rectangle	Rectangle 4 in. \times 2 in. Serrated figure as in Exp. 11	13 44	18	<0.01	5	20 each, 5 times
Figures of equal diameter; different complexity of contour	16-pointed star 3 in. diameter Circle 3 in. diameter	59 27	14	<0.01	5	20 each, 5 times
Figures of equal diameter; different complexity of contour	8-pointed star 3 in. diameter Circle 3 in. diameter	30 12	18	<0.01	5	10 each, 5 times

Statement of results

The results show firstly that in all cases (except Exp. 7) the insects orientated quite definitely to the black objects present.

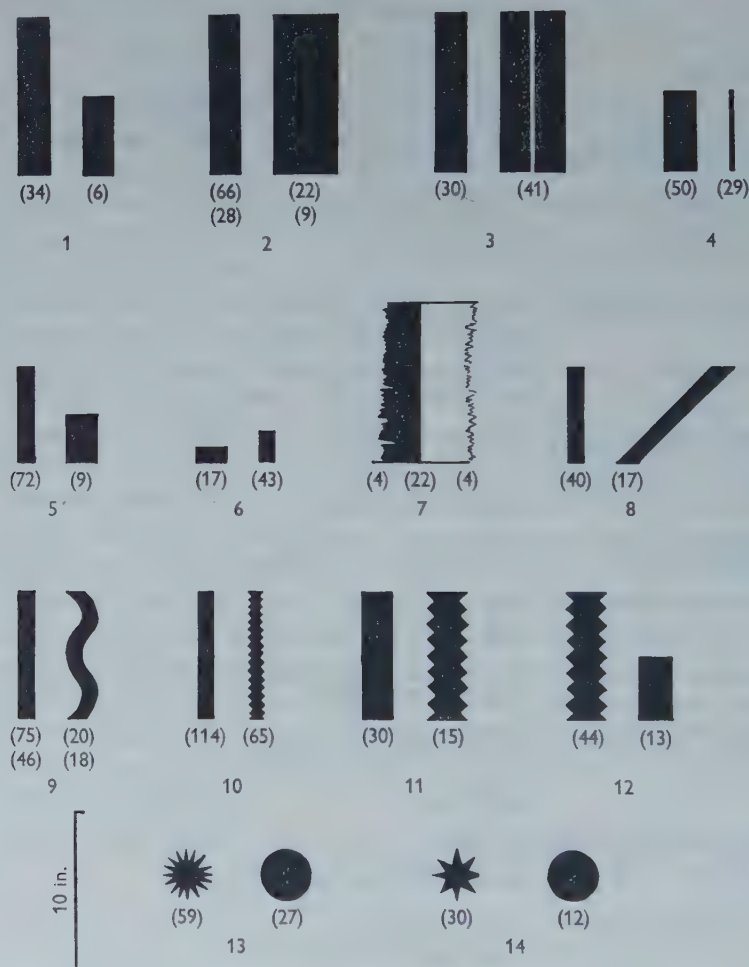


Fig. 1. Diagram summarizing the results of spontaneous choice experiments to study form discrimination in nymphs of the desert locust *Schistocerca gregaria*. The shapes are all to the same scale, which is shown. The single number below each pair of shapes is the number of the experiment. The number in brackets below each shape indicates the number of visits to that shape. Where there are two sets of bracketed numbers these are referred to as *a* and *b* in Table 1.

First experiment

In this experiment (rectangles of breadth 2 in.; one 10 in. tall, the other 5 in. tall) the insects showed a preference for the taller figure. It was not clear, however, exactly which properties of the tall figure made it more attractive. Apart from the

obvious feature of height the taller figure had also a greater area and perimeter than the shorter one. Exps. 2-7 were intended to evaluate the importance of these properties. These will now be discussed.

Area

The effect of area was tested in two ways:

- (1) By comparing figures of equal height and different area (Exps. 2-4).
- (2) By comparing figures of equal area and different height (Exp. 5).

The result of Exp. 2 (rectangles 10 in. tall; one 2 in. broad, one 4 in. broad) showed that in this situation it was not the figure of greater area which was chosen.

In Exp. 4 (rectangles 5 in. tall; one 2 in. broad, one 0.25 in. broad) the figure of greater area was chosen. This may, however, have been due to the fact that the smaller figure was extremely narrow.

Exps. 2 and 4 thus suggested that the effect of area was variable. Furthermore, it was found that the bias in favour of the narrower stripe in Exp. 2 could be significantly reversed by subdividing the broad figure with a vertical narrow white stripe (Exp. 3). Thus it appeared that not only was the effect of area variable but also that it could be outweighed by the number of vertical black/white edges.

In contrast to the variable effect of area, Exp. 5 showed that the effect of height was very marked. The figures were of equal area (rectangle 6 in. tall, 1 in. broad and rectangle 3 in. tall, 2 in. broad) and the taller was preferred.

Perimeter

Exps. 2-5 suggested that the effect of perimeter was also variable. Thus the chosen figure in Exp. 2 had a smaller perimeter than the alternative figure. In Exps. 4 and 5 the chosen figure had a greater perimeter than the other. It is significant that in these last two cases the greater perimeter is due to a greater length of vertical edge.

In Exp. 6 not only were the figures of equal area but also of equal perimeter (rectangles 2 in. \times 1 in.; one with long side vertical, the other with long side horizontal). The taller figure was chosen. The larger number of misses (30) was probably due to the small size of the objects. This small size was rendered necessary since the largest side could not be much greater than 2 in. otherwise the breadth of the horizontal figure would complicate matters. (This arose from Exp. 2, where it was found that a very broad object was not attractive, irrespective of height.)

Tall black/white edge

The experiments so far had demonstrated the variability of the effect of area and perimeter and emphasized the importance of tallness as an attractive feature. It was possible to be more specific and to say that it was the length of the vertical black/white edge which was important. In all the experiments the insects were seen to orientate to the vertical black/white edges when approaching the figures. Also, the addition of extra black/white edges (Exp. 3) increased the attractiveness of a

figure. (In this experiment the insects also orientated to the vertical black edges in the centre of the figure.) Finally, if the whole of the circumference of the arena were divided into equal panels of black and white (Exp. 7) the insects orientated to the vertical black/white boundaries.

Verticality

The importance of the verticality of the black/white edge was tested in Exp. 8. Both figures had straight edges but in one case the long edge was at 90° to the horizontal while in the other it was at 45° . Both figures were of the same vertical height (6 in.). An insect reaching the arena wall at any point within the horizontal projection of the oblique was counted as orientating to that figure.

The insects showed a significant preference for the vertical figure.

Straightness of edge

Exps. 9-11 were designed to evaluate the importance of the verticality of a figure as a whole as compared with the verticality of its edge. It is obvious that in one sense it is impossible to have an edge which is vertical if it is not straight. However, it is possible to have a figure which, as a whole, is vertical, i.e. with the long axis in the vertical plane, although the edges of the figure are not straight and therefore not vertical. In Exps. 9-11 all the figures were vertical in this sense and a comparison was made between a vertical figure with a straight edge and one with a wavy or serrated edge. The results showed that in these situations a straight edge was more attractive than a wavy or serrated edge.

In these experiments it should be noted also that the chosen figure was that with the smaller perimeter in each case.

It was clear also that area was of little importance in these cases. In Exp. 9 the negative figure was the larger in area, in Exp. 10 it was the smaller and in Exp. 11 the areas of the figures were equal.

Equating attractiveness of properties

The importance of the straight vertical edge had been clearly demonstrated in the preceding experiments. Exp. 12 showed, however, that the effect of this property could be outweighed by a preponderance of a different property in the alternative choice figure. In this situation the taller figure was preferred, although it had a serrated edge whereas the short figure had a straight edge.

Complexity of contour

Hertz found that, for bees, the amount of the contour was an important figural property. Exps. 13 and 14 tested the importance of this property for locust nymphs in situations where none of the figures possessed a straight vertical edge. Under these conditions the figure with the more broken-up contour was preferred.

DISCUSSION

Since the results reported here are similar in many respects to those obtained by previous workers for other insects it is unnecessary to compare them individually in detail and only the main results will be so considered. These are: (1) attraction to figure edges; (2) preference for vertical edges as against oblique, wavy or serrated edges; (3) importance of complexity of contour.

Attraction to figure edges is well known. Kalmus (1937) described it in the nymphs of *Dixippus morosus* and called the response 'photo-horotaxis' (see also Hundertmark, 1937*b*). Certain lepidopteran larvae (Hundertmark, 1937*a*) and sawfly larvae (personal observation) also show the response, as do certain grasshoppers (Williams, 1954).

Preference for a vertical edge as opposed to an oblique edge is shown by *Lymantria* caterpillars (Hundertmark, 1937*b*). Williams found that, although his grasshoppers did not show any preference for a vertical stripe as against a vertical zig-zag of stripes, nevertheless they did show a preference for vertical stripes as against horizontal stripes. The present results also show that the vertical edges of the figures are more important than the horizontal edges.

Hertz (1929*b*, 1931, 1933) and Zerrahn (1934) showed that, for bees, the more complex the contour the more attractive the figure. Ilse (1932) showed the same for certain butterflies.

It is fairly well established that in any theory of form discrimination in insects the operative stimuli to be considered are the changes in the intensity of light falling on the ommatidium when an image moves on the retina (Hertz, 1929*a, b*, 1931, 1933, 1934*a, b*, 1935, 1937; Wolf, 1935; Autrum, 1948, 1952). Henceforth, for shortness, such changes will be referred to as 'stimulus changes'.

Before proceeding with the discussion the following point must be made with regard to the units involved. Underlying most previous work on insect vision has been the mosaic theory of image formation by the apposition eye (Müller, 1829, in Wigglesworth, 1953). This theory supposes that each ommatidium receives light from only a very limited region of the visual field. Thus 'each ommatidium receives the impression of a luminous area corresponding to its projection on the visual field' (Wigglesworth, 1953). Previous workers have interpreted their findings in terms of the visual field of the ommatidium as strictly equivalent to the projection of the ommatidial angle (e.g. Baumgärtner, 1928; Wolf, 1935; Gavel, 1940; Hassenstein, 1951). It has been shown, however, by Burt & Catton (1954) that in *Locusta migratoria migratorioides*, and in the hive bee that the visual fields of the ommatidia overlap to a considerable extent, and these authors suggest that this is probably true of most compound eyes. It is desired to point out here that the argument which follows is not affected in principle by this factor of overlap since, whatever the functional units, be they single ommatidia or groups of ommatidia, their distribution will be determined by the distribution of the ommatidia in the eye.

Many of the results of the present experiments are immediately explicable on the basis that the insects are attracted to the figure whose contour produces the

greatest number of such changes per unit time as it moves past the eye. Clearly the vertical contours will be the important ones since they are perpendicular to the direction of the insect's motion when walking on a horizontal surface* and will thus produce the greatest number of stimulus changes per unit time for any movement of the insect. Figures with longer vertical contours will be more attractive than those with shorter ones (Exps. 1, 5 and 6). If the lengths of the vertical edges are equal an increase in the number of vertical contours will increase the attractiveness of a figure (Exp. 3. Compare experiment and control). Complexity of a figure contour will also increase the number of stimulus changes per unit time and thus increase the attractiveness of the figure (Exps. 13 and 14).

A difficulty arises in the case of Exps. 8-11. It is probably justifiable to consider that, to a first approximation, the number of ommatida stimulated by the movement of a contour is proportional to the area 'swept out' by the contour. Thus if a figure moves a certain distance X in a straight line the area swept out by its leading contour is LX , where L is the length of this contour. This will be the area swept out by a vertical contour of length L moving a distance X horizontally. Considering an oblique contour moving the same distance horizontally, it can be shown that if the oblique contour is of such a length as to reach the same vertical height as the vertical contour then it will sweep out the same area as does the latter; i.e. it will stimulate the same number of ommatidia. Hence, it would appear that the vertical and oblique figures in Exp. 8 should stimulate the same number of ommatidia for the same movement over the eye. Thus, purely on this basis they should be equally attractive. The same argument applies in the case of the wavy and serrated edge (Exps. 9-11). Yet in each case the vertical edge is chosen. These experiments therefore suggest that, apart from the number of receptors stimulated, special significance attaches to the spatial distribution of the receptor cells which are stimulated at any particular instant. To be more specific, an important feature of the stimulus may be the proximity of the groups of ommatidia which are stimulated. It is possible that, apart from three unique orientations, the passage of the image of an oblique edge over the retina may result in more spatial and temporal dispersion of the ommatidia which are stimulated than is the case for a vertical contour. (The unique directions are a consequence of the hexagonal nature of the ommatidium and are the directions in which a straight line will pass through the centres of adjacent ommatidia—see Gavel (1940) and Hassenstein (1951). The pattern of the ommatidial projections will also have this feature.)

The results of Exp. 2 remain to be considered, for it is clear that they are not explicable on the bases already postulated. The long contours of both figures are vertical and of equal length so that they will stimulate the same number of ommatidia in the same spatial pattern. The previous postulates are conceived in terms of ommatidia undergoing simultaneously changes of the same 'sign', i.e. all either responding to an increase in light intensity (going 'on') or to a decrease in light

* This motion of contours over the eye will be produced also by the peering movement already described. A detailed account of this movement and its significance will be published elsewhere. It is relevant to state here that there is evidence to show that it is a scanning movement.

intensity (going 'off'). With respect to Exp. 2 it can be suggested either that the spatial distribution of ommatidia which are stimulated simultaneously whatever their 'sign' is important or that the shortness of the time interval between the 'on' and 'off' state of any one ommatidium is important. These features are not mutually exclusive, and both depend on the distance between the moving contours, i.e. on the breadth of the figures.

Hertz (1929*b*) advanced the opinion that a spontaneous choice of certain figures, such as she had found to occur in bees, would also be found in other arthropods with different living requirements, and that these would reflect the properties of a simple nervous system. It is interesting, therefore, to consider the present results in the light of the theories of form discrimination which have been put forward in the case of the bee (Hertz, 1929*a*, 1931, 1933, 1934*a, b*, 1935, 1937; Wolf, 1934; Zerrahn, 1934; Wolf & Zerrahn-Wolf, 1934, 1936).

The view of Wolf & Zerrahn-Wolf can be stated very briefly. They believed that the bees were attracted to the figure which caused the greatest number of stimulus changes per unit time as the bee flew over it and that only this total number was important. They found that, in the figures studied, there was a linear relationship between the percentage choice of a particular figure and its contour length. They concluded therefore that 'recognition and distinction of patterns by bees is based upon the transitory stimulation produced by a pattern and that the pattern as such is of no importance' (Wolf & Zerrahn-Wolf, 1936).

Hertz (1931, 1935) denied that this was a complete explanation. She herself distinguished two main classes of figural property which determined a figure's attractiveness and on which figures were differentiated. These were figural intensity and figural quality. Figural intensity was, in effect, number of stimulus changes per unit time. Figural quality depended on the spatial rate of change of angle of a contour line, the greater this rate of change the more attractive the figure. Figures such as small circles with strongly curved contours or those with serrated contours possessed this quality (Hertz, 1933).

As has been discussed above, a theory based on number alone does not seem to be the complete explanation of form discrimination in the desert locust, and it has been suggested that the spatial and temporal separation of the ommatidia which are firing is also important. The importance of this spatial and temporal distribution was pointed out by Hertz (1935), and it therefore seems that the present findings are more in general agreement with her theory than with that of Wolf & Zerrahn-Wolf. The present results do, however, appear to differ in detail from what would be expected on Hertz's theory, for on this latter basis wavy and serrated figures should possess greater figural quality than the vertical ones and should be preferred to them.

The present findings suggest that different criteria are used for form discrimination in different situations depending on the nature of the figures involved. It seems that whenever the figures produce greatly differing numbers of stimulus changes per unit time the discrimination is made on this basis. (Hence in Exp. 12 the attractiveness of the taller stripe is greater than that of the shorter vertical stripe.)

It should be noted also that where there is such a large difference between the figures the preference in each case is strongly marked (see Exps. 1, 5). When figures produce approximately equal numbers of stimulus changes per unit time it seems that the discrimination may be made on the basis of the spatial and temporal proximity of the ommatidia involved. The results of such experiments suggest that this discrimination is perhaps less readily made.

SUMMARY

1. Nymphs of the desert locust are spontaneously attracted to simple forms in the visual environment, and show a preference for certain figures.
2. Experiments are described which analyse certain of the important properties which make a figure attractive to this insect. The insects show a preference for long straight vertical edges as opposed to short straight vertical edges. Straight vertical edges are preferred to straight oblique edges and vertical figures with straight edges are preferred to vertical figures with wavy or serrated edges. In the absence of straight vertical edges a preference is shown for the figure with the more complex contour (for figures of comparable size).
3. It is postulated that in this insect form discrimination is based on the number of stimulus changes per unit time produced by moving contours and on the spatial and temporal distribution of such changes.
4. The results are compared with those of previous workers and in particular with those of Hertz and of Wolf and Zerrahn-Wolf on the bee.

The experiments reported in this paper were performed during the tenure of grants from the Carnegie and Cross Trusts and formed part of a thesis for the degree of Ph.D. of St Andrews University. I wish to thank Prof. G. C. Varley for his kindness in affording me facilities in his department and P. Hunter-Jones of the Anti-Locust Research Centre for supplying the locusts. I am grateful to F. L. Waterhouse, Queen's College, Dundee, for his criticism and encouragement and to E. R. F. W. Crossman and D. M. Vowles of the Psychology Department, University of Reading, for their criticism of this manuscript.

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FLIGHT ACTIVITY IN THE BLOWFLY, *CALLIPHORA ERYTHROCEPHALA*, IN RELATION TO WIND SPEED, WITH SPECIAL REFERENCE TO ADAPTATION

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(Received 18 April 1958)

INTRODUCTION

Wind speed is prominent among the various weather factors which affect an insect about to take to flight.

Field studies have suggested that wind inhibits the flight activity of, for instance, the desert locust (Waloff & Rainey, 1951), the red locust (Rainey, Waloff & Burnett, 1957), and the tsetse fly (Swynnerton, 1936). On the other hand Vanderplank (1948) found no correlation, and Johnson (1954) a variable one, between wind speed and the catches of the tsetse fly and of aphids respectively. Observations on the activity of locusts early in the morning (Strelnikov, 1936; Kennedy, 1939; Waloff & Rainey, 1951) have suggested that wind may sometimes have an activating effect. The effect of wind in the field is clearly complex and is difficult to interpret as it is usually associated with changes of other factors, in particular of temperature.

Laboratory experiments have shown that wind inhibits flight. Thus Davies (1936) showed this effect in the aphid *Myzus persicae*, and Haine (1955*a, b*) has confirmed these observations and extended them to a number of other aphids. The experiments by Davies (1936) did not show adaptation to wind during the 10 min. periods of exposure to the high wind speed, but experiments by the author indicated that adaptation occurred in the reactions of the blowfly *Calliphora* to wind.

The occurrence of adaptation is important, for wind under the conditions in which insects usually take to flight is normally turbulent, and the effect of wind on activity will therefore depend largely on the rapidity of the reaction to a change of wind and on the extent and rate of any subsequent adaptation.

A study of the flight activity of the blowfly *C. erythrocephala* Mg. in relation to light and radiant heat showed that while individual activity is variable the average activity of a group is relatively uniform and may be related to stimulus with some degree of confidence (Digby, 1958). Further wind-tunnel experiments were therefore carried out in an attempt to clarify the nature of the reaction to wind and the part played by adaptation.

METHODS

General methods

All observations were carried out on groups of flies confined in a celluloid chamber in a small wind tunnel, by procedures similar to those described previously (Digby, 1958). Groups of forty flies between 2 and 6 weeks of age were used for most

experiments. The use of younger flies was avoided because their activity in the chamber often consisted of periods of continued flight and was therefore difficult to assess, and the use of older flies was avoided because their activity tended to fall to a low level and to become very irregular. Unless otherwise stated, the relatively low light intensity of 160 lux was used in order to avoid the possibility of temperature changes due to radiation interfering with results at low wind speeds. The mechanical vibration produced by the fan was found to reduce activity, the flies becoming rather sensitive to it after a period under still conditions. Wind velocity was therefore altered by adjusting the various shutters (shown in fig. 1 of Digby, 1955), while the motor speed was maintained constant throughout a given series of experiments. All experiments were run at the temperatures and humidities of the laboratory, which ranged from 14.7° to 23.7° C. and from 46 to 80 % R.H. between experiments. All but a few experiments were carried out between 18° and 23° C. Temperatures were maintained constant to within 1.0° C. during any one experiment, and the insects were adapted to laboratory temperature and humidity for several hours before each series of experiments.

Measurement of wind speed

The hot-wire anemometer was used for speeds below 1.0 m./sec., and above this speed the Pitot tube was employed. The manometer attached to the latter was filled with chloroform and tilted so as to give a large-scale reading for a small pressure difference. The scale was calibrated by using a previously calibrated fan anemometer as a standard. The use of still air was avoided in order to guard against undesirable variations of temperature. A slow drift of air of about 0.05 m./sec. was therefore the minimum used during observations on activity. This speed could be measured readily with the hot-wire anemometer, and caused a deflexion of about 3 mm. on the meter used in the bridge circuit.

Insects at rest on the transparent sides of the insect chamber experienced smaller wind speeds than those measured by the anemometer in the centre of the wind channel. The average wind profile close to the surface is shown in Table 1 for the cross-section passing through the centre of the chamber. The measurements were obtained by using the hot-wire anemometer or Pitot tube as a probe. At low wind speeds the upper and lower surfaces of the thorax of a resting fly were respectively 0.5 and 0.25 cm. from the surface. The insects crouched closer to the surface at higher speeds. Most of the flies were therefore exposed to a gradient of wind speed ranging from that measured by the anemometer to a value about 30 % less, while a proportion immediately down-wind from the others were sheltered to a varying degree. The wind speeds quoted in the following experiments are for convenience the values measured by the anemometers in the usual position near the centre of the wind channel.

The range of nominal wind speed to which the flies were exposed covers that found in the field. Measurements of wind speed in vegetation are usually below 1.0 m./sec. and an insect at rest is rarely in a position where it is exposed to a wind so great as 8.0 m./sec.

Table 1. *Reduction of wind speed close to surfaces and corners of insect chamber for cross-section in middle of chamber*

Position		Wind speed (m./sec.)		
Centre of cross-section		0.5	4.5	8.0
From mid-points of surfaces	1.0 cm. from surface	0.50	4.4	7.5
	0.5 cm. from surface	0.49	4.1	7.0
	0.25 cm. from surface	0.39	3.4	6.7
From corners inwards at 45° from the surfaces	1.0 cm. from corner	0.5	4.3	7.5
	0.5 cm. from corner	0.45	3.8	6.2
	0.25 cm. from corner	—	3.3	5.3

Measurement of activity

Flight activity was assessed exactly as described previously (Digby, 1958), that is by counting the total number of short flights in 45 sec. out of every minute and reducing the figure to flights/specimen/min. On some occasions walking activity also was assessed towards the end of each minute period by counting the numbers which were walking continuously or with short rests of 1 or 2 sec., at the moment of observation. When more active, the numbers walking were estimated to the nearest five. The figures are expressed in this paper as the percentage of the population showing walking activity.

THE EFFECT OF WIND SPEED

Flight and walking activity

In order to study the relation of activity to wind, groups of flies were subjected to a convenient standard wind speed of 0.5 m./sec. for a period of 30 min. or longer, to ensure a reasonable degree of adaptation. The wind speed was then changed to a higher or lower value for a further period, after which it was returned to the standard speed once more. Three such experiments, shown in Figs. 1-3, illustrate the changes from 0.5 m./sec. to 3.0, to 8.0 and to 0.05 m./sec. The change to both higher and lower speeds resulted in reduction of flight activity. This was most strongly marked at the higher wind speeds, and here the decrease of flight activity which occurred when the wind speed was raised to a high value was followed by a partial recovery as the insects adapted to the higher level of stimulus. On return from the high value to the standard 0.5 m./sec. activity showed an immediate increase, the rate at first rising to a level much greater than that during the initial period at the standard wind speed, and then declining as adaptation occurred back to a level of activity appropriate to the 0.5 m./sec. speed. Adaptation effects were very slight at low wind speeds (Fig. 3).

Walking activity was affected by change of wind speed in a way closely parallel to flight activity. An estimate of the percentage walking during the change to 8.0 m./sec. and back again is shown in Fig. 2, the observations on walking and on flight activity being taken from two identical experiments. The change of activity due to the change of wind from 0.5 to 0.05 m./sec. and back (Fig. 3) was relatively

small and difficult to follow in these experiments where the flies were adapted to a wind of 0.5 m./sec.; but if they were adapted to still air and observed at a low light intensity the walking activity was much less and therefore more easy to follow, the effect of the change of wind speed being greatly enhanced. Fig. 4 shows one such

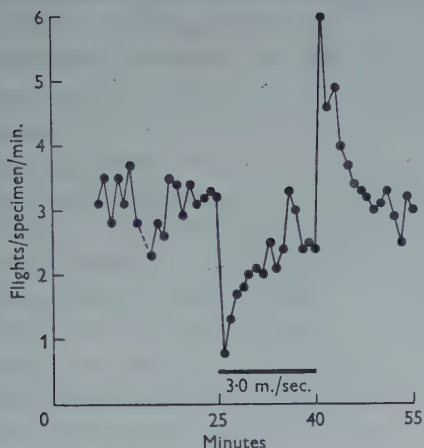


Fig. 1

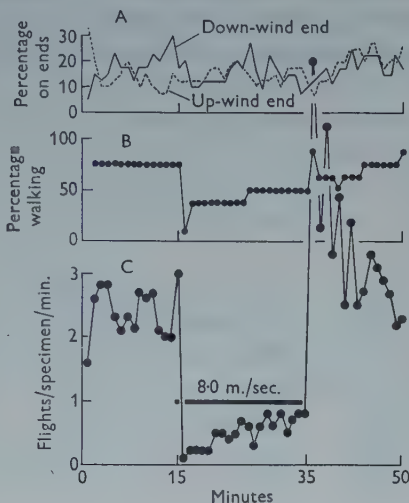


Fig. 2

Fig. 1. Effect of change of wind speed from 0.5 to 3.0 m./sec. and back on mean flight activity of a group of forty flies.

Fig. 2. Effect of change of wind speed from 0.5 to 8.0 m./sec. on: A, percentage on up-wind end of insect chamber (dotted line) and down-wind end (continuous line); B, percentage walking; and C, flight activity. Data from two identical experiments.

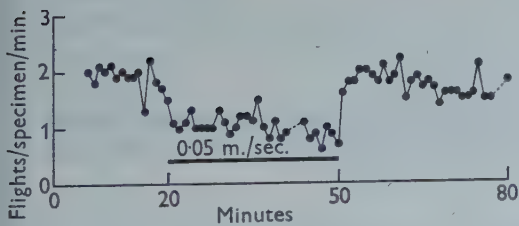


Fig. 3

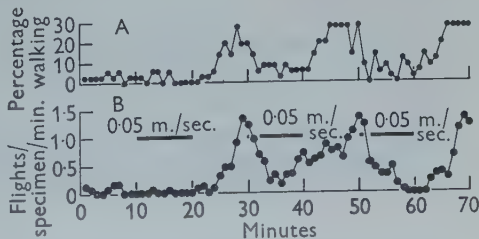


Fig. 4

Fig. 3. Effect of change of wind speed from 0.5 to 0.05 m./sec. and back on flight activity.

Fig. 4. Effect of change of wind speed from 0.5 to 0.05 m./sec. on: A, walking activity; and B, flight activity in the same experiment. Run at low light intensity (2.0 instead of 160 lux).

experiment carried out at a light intensity of 2 lux with insects previously adapted to still-air conditions. It can be seen that there was a very close agreement between walking and flight activity.

Curves relating the change of flight activity to change of wind speed are shown in Fig. 5. The curves were produced from the results of ninety-four experiments,

similar to those of Figs. 1-3 but mostly of shorter duration, carried out at 160 lux. Flight activity for the periods of the first, the second to the fifth and in a few cases of the twenty-first to thirtieth minutes after the change of wind speed from 0.5 m./sec. is expressed as a percentage of that prevailing during the last 10 min. before the change. The distribution of the points shows that under these particular conditions the optimum wind speed for flight activity lies at about 0.7 m./sec. There was a considerable degree of adaptive recovery from the inhibiting effects of a continuing

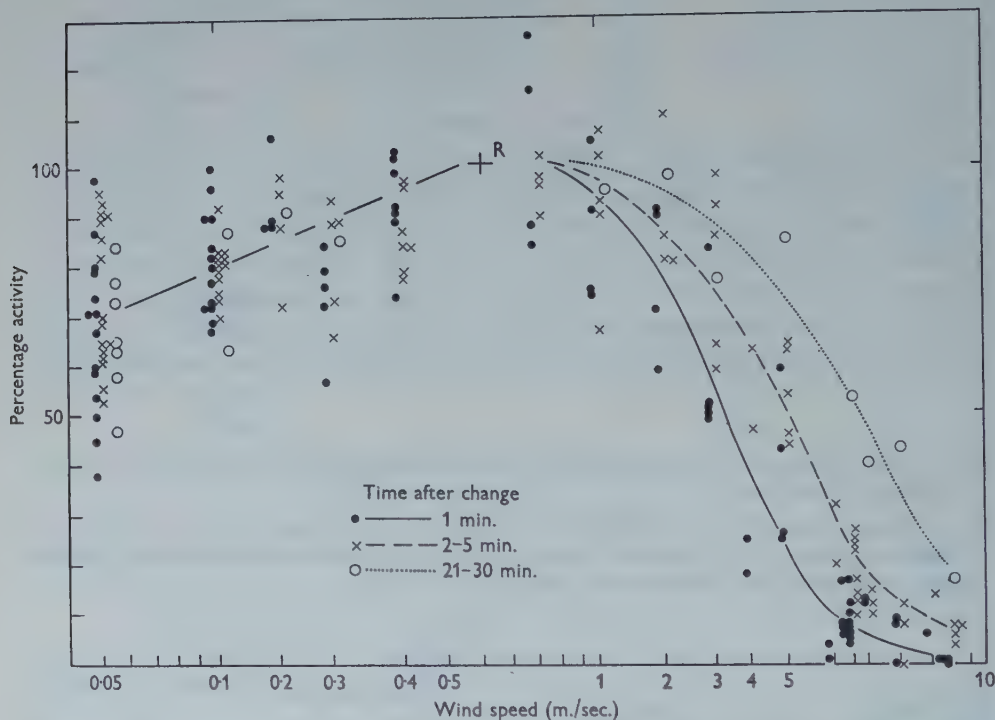


Fig. 5. Activity following a change of wind speed at various periods after the change. Activity expressed as a percentage of that prevailing during the last 10 min. of the preceding period at 0.5 m./sec. The point *R* is the reference point, 100 % at 0.5 m./sec.

high wind, but the adaptive changes which followed reduction of the wind speed from 0.5 m./sec. to lower values were almost negligible in these experiments. There was a considerable range in the degree of sensitivity to low wind speed. This sensitivity was readily lost after prolonged activity or prolonged exposure to a wind of 0.5 m./sec., or after a short exposure to a strong wind.

The temperatures and humidities at which these experiments were carried out ranged from 16.6° to 23.4° C. and from 46 to 80% R.H. There was no significant correlation between these factors and activity change either above or below the optimum. Nor was there a significant correlation with the initial level of activity, which varied from 1.3 to 4.2 flights/specimen/min.

Mechanical effects of wind speed

The question arises to what extent the flight activity as observed reflects the true excitability of the population and to what extent it is modified by the insects either being blown off the surface or being unable to leave it. At the standard wind speed of 0.5 m./sec. the insects were distributed more or less at random and flights were made about the chamber in all directions. Observation suggested that at this speed and below it almost every flight was voluntary. Furthermore, when the wind speed was reduced to 0.05 m./sec., the proportion walking, and also the tempo of activity displayed in walking, decreased together with the flight activity (Fig. 4), showing the activating effect of a wind speed of 0.5 m./sec. to be a true activation and not a spurious effect arising from the insects being dislodged from the walls of the chamber by a stronger wind.

Above 0.5 m./sec. flight resulted in displacement down-wind more commonly than up-wind. At 4.5 m./sec. up-wind flights were still possible, but activity on the gauze at the down-wind end was restricted in the main to short hops of an inch or two. At this speed, all the members of the group appeared to be able to make short hops and flights, which could easily be seen and counted. It is therefore unlikely that mechanical inability to take to flight played any significant part in modifying flight activity at 4.5 m./sec. and observation suggested that nearly all the flights were still voluntary.

At the highest speed employed, 8.0 m./sec. (Fig. 2), the insects initially at rest or walking on the sides or on the up-wind end of the chamber were swept down-wind immediately they took to flight or left the surface. It was not possible to see whether all these flights were voluntary in origin, but the fact that walking and flight activity in normal flies was reduced almost to zero for a minute or two after increasing the wind to 8.0 m./sec. showed that the insects at rest were not swept off the surface by the force of the wind even at this speed. After the initial great reduction of activity the proportion walking, and the activity displayed in walking, increased again together with the flight activity. A part of the apparent flight activity during this period may well be due to the insects losing their footing whilst walking in the strong wind. Most of the flies on the down-wind end, usually about one-third of the total number in the chamber, were unable to make hops or flights away from the surface at this speed.

It may therefore be concluded that the changes in flight activity described in these experiments concern mainly activation or inhibition of flight activity up to 4.5 m./sec. and mechanical factors arising from the force of the wind play a part in modifying this activity at 8.0 m./sec.

The position of the population in the insect chamber

The effect of high wind speed on the distribution of the flies within the chamber is of significance to the estimation of flight activity, for the insects on the down-wind end could not make flights at 8.0 m./sec. The population of the whole, however,

remained fairly well distributed about the chamber in the face of very considerable changes of wind speed.

In these experiments when the flight activity was about 2.0 flights/specimen/min. roughly half the flies were in the walking phase of activity at 0.5 m./sec. The direction of walking, as of flying, was more or less random. When the wind speed was increased the numbers walking decreased; but a greater proportion of the insects became orientated into the wind. This resulted in their walking up-wind at a rate which tended to counteract the rate of down-wind movement of the population resulting from flight.

A count of the numbers on the down-wind end was taken as a measure of the extent to which these two movements balanced. Table 2 gives the results of 8 experiments in which the wind speed was changed from 0.5 m./sec. to a higher value for 15 min. or more. One of these is that shown in Fig. 2. Up-wind movement predominated when the wind was changed to 2.0 and to 4.5 m./sec. The two tendencies balanced remarkably well, and not more than 20% of the population was found on the down-wind end at the speed of 8.0 m./sec. at which flight activity was limited for mechanical reasons.

Table 2. *Normal flies; proportion on down-wind end of insect chamber after change of wind speed from 0.5 m./sec. to higher values*

Exp. no.	Higher value of wind speed	Percentage on down-wind end						
		During last 10 min. of initial period at 0.5 m./sec.	During following periods after change to higher value (min.)					
			1-5	6-10	11-15	16-20	21-25	26-30
1	8.0	27	15	11	16	19	11	6
2	8.0	20	18	9	17	16	—	—
3	8.0	20	13	16	19	12	—	—
4	6.5	20	24	23	31	41	40	39
5	4.5	19	22	25	22	22	—	—
6	4.5	20	29	32	36	33	—	—
7	2.0	15	32	18	20	19	—	—
8	2.0	22	25	33	37	—	—	—

THE ANALYSIS OF ADAPTATION TO WIND

The range of wind speed from 0.5 to 4.5 m./sec., in which an increase of wind speed decreases activity

Adaptation effects were prominent in the relation of activity to wind above 0.5 m./sec. Analysis of adaptation to higher and to lower wind speeds was carried out in the range from 0.5 to 4.5 m./sec. This range was notable for the powerful inhibition produced by a strong wind; the small activating effect included may be neglected.

The methods used were those described for studying adaptation to change of light intensity (Digby, 1958). The principle is that the extent of rebound during the first minute after a period at a different level of stimulus may be taken as a measure of

the amount of adaptation to it. Two sets of experiments were carried out. In the first set (Fig. 6) an initial observation of activity was made over the last 10 min. of a period of $\frac{1}{2}$ hr. or more at 0.5 m./sec.; the wind speed was then changed to 4.5 m./sec. for a period which was varied in successive experiments and lastly was returned to 0.5 m./sec. Observations of activity were made in this second period at 0.5 m./sec., the activity being expressed as a percentage of that during the last 10 min. of the initial period at this speed. The recovery curves correspond to the continuous line of the inset graph (Fig. 6). They show the time course of the adaptive decline of activity which followed the immediate peak caused by the cessation of inhibition due to the high wind speed. The distance of the initial

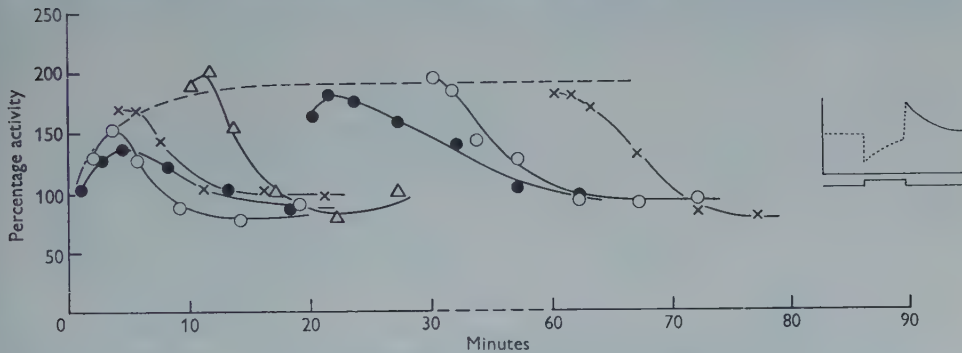


Fig. 6. Higher wind range; adaptation to high wind speed (i.e. family of recovery curves at 0.5 m./sec. after varied periods at 4.5 m./sec. during which the activity was much less; see inset graph). The broken line passing through the initial point of each recovery curve shows the time course of adaptation to 4.5 m./sec. Activity expressed as a percentage of that prevailing during the last 10 min. of the initial period at 0.5 m./sec., time as minutes elapsed since the end of this initial period.

point of each recovery curve along the time scale marks the duration of the preceding period of high wind speed. The initial points of the recovery curves depart from the 100 % level by an amount which is a measure of the amount of adaptation to the 4.5 m./sec. wind speed at that instant, and the broken line passing through the initial points indicates the course of this adaptation in relation to time. This curve is diagrammatic since, as in the case of those shown for adaptation to light, the population varied somewhat in sensitivity between the experiments.

In the second series of experiments (Fig. 7) the changes were carried out with the wind velocities in the reverse order, the family of recovery curves at 4.5 m./sec. being observed after a period at the lower wind speed during which the activity had been much greater. These curves show the adaptive increase of activity which followed the immediate depression resulting from resumption of the high wind speed. In this case the departure of the initial point of each recovery curve from the initial level of activity is a measure of the extent of adaptation to the lower wind speed, and the broken line indicates the course of adaptation to the lower wind speed in relation to time.

These two sets of curves show that a longer period of adaptation was followed by a slower recovery from the subsequent rebound and that adaptation to both the higher and the lower wind reached a maximum after 10–20 min. The one set of curves bears a close resemblance to the other set inverted, the increase of activity following the release of inhibition (Fig. 6) being nearly as sharp as the decrease of activity which occurred when the inhibition was applied (Fig. 7). In this there is a striking contrast to the adaptation phenomena described for increase and decrease in light intensity, where the increase of activity was delayed by the persistence of a state of low activity. A trace of the same phenomenon can be seen here, however, in that the peak of activity following the removal of inhibition by a 4.5 m./sec. wind (Fig. 6) is reached in the earlier curves not in the first minute after the change, but in the second to third minutes after.

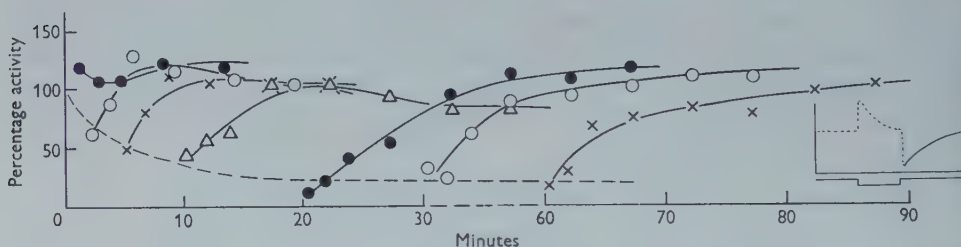


Fig. 7. Higher wind range; adaptation to low wind speed (i.e. family of recovery curves at 4.5 m./sec. after varied periods at 0.5 m./sec. during which activity was much greater; see inset graph). The broken line passing through the initial point of each recovery curve shows the time course of adaptation to 0.5 m./sec. Activity expressed as a percentage of that prevailing during the last 10 min. of the initial period at 4.5 m./sec., time as minutes elapsed since the end of this initial period.

*The range from 0.05 to 0.5 m./sec., in which an increase of
wind speed increases activity*

Comparable experiments were carried out in this range. Recovery curves at 0.05 m./sec. after periods of up to 1 hr. at 0.5 m./sec. were all very similar, showing activity to decline to the level appropriate to the lower wind speed during the first minute and then to remain steady as in Fig. 3. The lack of subsequent recovery can be seen in Figs. 3 and 5.

In contrast, recovery curves at 0.5 m./sec. after various periods at 0.05 m./sec. (Fig. 8) all show a much slower increase of activity up to the level appropriate to the 0.5 m./sec. wind, taking more than 10 min. to obtain a steady value after an hour at the lower wind speed. As before, the longer the initial period at 0.05 m./sec., the slower the recovery. These curves are superficially similar to those obtained at 4.5 m./sec. after varied periods at 0.5 m./sec. (Fig. 7), but this similarity is only superficial since in the latter case the curves are those of recovery from an immediate rebound from a much higher level of activity occurring during the previous period at the lower wind speed. The recovery curves after varied periods in still air are more comparable to those following an increase of stimulation by light intensity (Digby, 1958, Fig. 7), showing the same slow recovery from a state of low activity.

The wind-adaptation curves differ from the light-adaptation curves in the absence of a conspicuous early peak of high activity, suggesting that adaptation to the stimulus of wind was much less than that to light at the values employed.

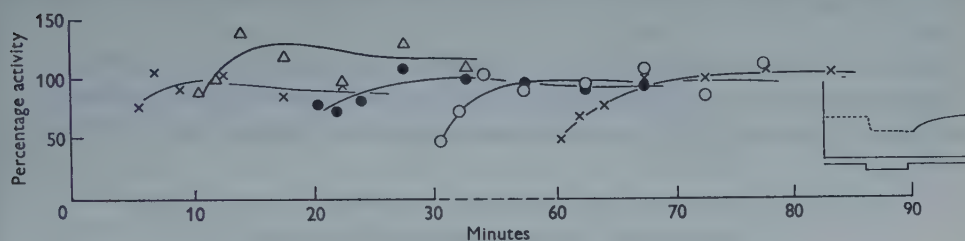


Fig. 8. Lower wind speed range; recovery curves at 0.5 m./sec. after varied periods at 0.05 m./sec., during which activity was less (see inset graph).

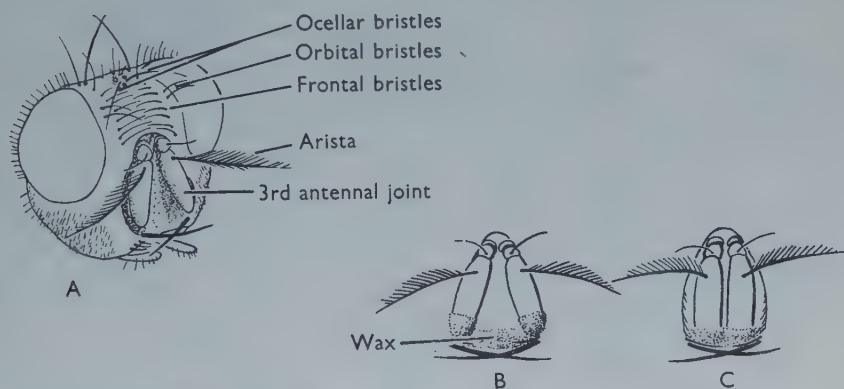


Fig. 9. Head of *Calliphora*. A, Normal fly, ♀; B, facial depression and antennae, waxed as control to experiments in which antennae immobilized; C, antennae immobilized by waxing to facial depression.

THE COMPONENTS OF THE REACTIONS TO CHANGE OF WIND SPEED

The inhibiting effect of antennal movement

The occurrence of an optimum wind speed with a decline of activity at higher and at lower speeds suggested that there might be at least two antagonistic reactions involved.

It is convenient to consider first the inhibition of activity to wind shown by normal flies above the optimum speed. A current of air causes movements of the antennae and of the numerous bristles distributed about the head and other parts of the insect. Each antenna consists of two short basal joints and an elongate third joint, which may lie vertically in the facial depression, or may be held out obliquely forwards (Fig. 9). This third joint bears a prominent plumose bristle, the arista. A current of air of about 0.5 m./sec. from the front of the insect moves the arista relative to the third joint to a rather limited degree, and it moves the third joint relative to the head, rotating it about its long axis. In stronger winds the third

antennal joint is deflected upwards and outwards. At the base of each antenna is a sense organ, Johnston's organ, situated in such a position as to suggest an ability to appreciate movements of the antennae. As the antennae are known to be concerned in flight reflexes (Hollick, 1940) it seemed reasonable first to investigate this organ.

In a series of experiments, details of which are given in Table 3, the two third antennal joints of each member of groups of forty flies were fastened into the facial depression with wax (Fig. 9), applied with an electrically heated loop of wire while

Table 3. *Effect of change of wind speed from 0.5 to 4.5 m./sec. on activity of flies with antennae immobilized*

(This change produces inhibition in normal flies.)

Exp. no.	Light intensity (lux)	Activity during preceding 5 min. at 0.5 m./sec. for each trial (flights/specimen/min.)	Effect of change of wind 0.5-4.5 m./sec. O, no effect; A, activation	
			On flight activity	On percentage walking
1	2600	2.62	O	O
		2.84	O	A
		2.20	O	A
2	2600	1.30	O	—
		1.28	O	—
		1.10	O	—
		1.16	O	—
		1.16	O	—
3	2600	1.27	O	A
		0.87	A	A
		0.17	A	A
4	2600	0.86	O	—
		1.24	O	—
		1.16	O	—
5	2600	0.56	O	A
		0.23	A	A
6	2600	0.53	O	A
		<0.08	A	A
		<0.03	A	A
7	160	1.52	O	O
		1.00	O	O
		0.74	O	O
8	2.0	0.65	A	A
		0.34	A	A
		0.64	A	A
9	160	0.43	A	—
		0.22	A	A
		0.22	A	A
10	2.0	0.22	A	A
		0.08	A	A
11	20.0	0.12	A	A
		0.13	A	A
		0.21	A	A
12	2.0	0.06	A	A
		0.02	A	A
		0.03	O	A

the insects were anaesthetized with a mixture of chloroform and ether. After recovery they were tested for the effect of the change of wind speed from 0.5 to 4.5 m./sec. Each experiment consisted of an initial acclimatization period of 10 or 20 min. at 0.5 m./sec. followed by alternate periods of 10 or 15 min. at 4.5 and 0.5 m./sec., each change from 0.5 m./sec. to the higher value constituting a trial. Each experiment was normally accompanied by a similar control run with previously anaesthetized but otherwise normal flies. Fastening the antennae to the head was found to abolish completely the inhibiting effect of a wind of 4.5 m./sec. (Fig. 10) and, when the activity was low, activation appeared instead (Fig. 11, Table 3). This operation also reduced the activity at 0.5 m./sec. by about one half, but the presence or absence of inhibition by a high wind was not related to the level of activity.

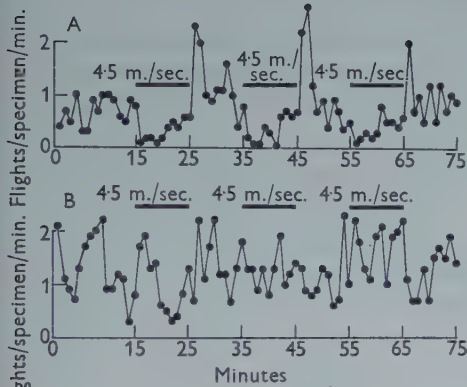


Fig. 10

Fig. 10. Effect of immobilizing antennae on reaction to change of wind from 0.5 to 4.5 m./sec. A, Control group, antennae waxed but not immobilized; B, experimental group with antennae immobilized.

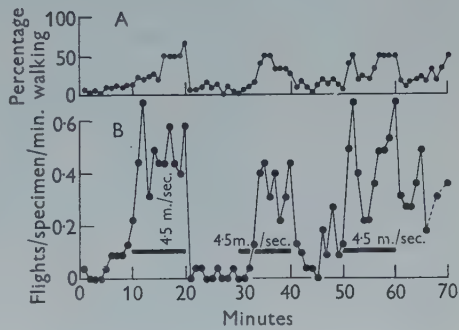


Fig. 11

Fig. 11. Effect of immobilizing antennae on reaction to change of wind from 0.5 to 4.5 m./sec. at low light intensity (2.0 lux). A, Walking activity; B, flight activity from same experiment.

In order to separate the possible effect of waxing the facial depression and antennae from the effect of immobilizing the antennae, the flies in four of the control groups, each numbering thirty or forty individuals, were waxed as in the experimental groups but the antennae were left freely mobile. A total of eleven trials with these groups showed a reduction of activity in a 4.5 m./sec. wind very similar to that shown by normal insects.

The arista is carried on the third antennal joint and as this projects into the air stream it seemed likely that much of the movement of the third joint might be due to deflexion of the arista by the wind. Amputation of the arista close to the base in each member of groups of twenty and thirty-three flies, without waxing down the antennae, had however a negligible effect, the population exhibiting a reduction of activity very similar to that of normal flies when subjected to a wind of 4.5 m./sec. Observation of single flies showed that amputation of the arista had in fact relatively

little effect on the upward and outward movement of the third joint of the antenna in a strong wind, although it abolished the slight rotation imparted by the arista at lower wind speeds. The arista therefore has little influence on the inhibiting effect of a 4.5 m./sec. wind.

Table 4. *Flies with antennae immobilized; proportion on down-wind end of insect chamber after change of wind speed from 0.5 to 8.0 m./sec.*

(Average of 8 expts.)

Percentage on down-wind end			
During last 10 min. of initial period at 0.5 m./sec.	During following periods after change to higher value (min.)		
	1-5	6-10	11-15
19	32	23	21

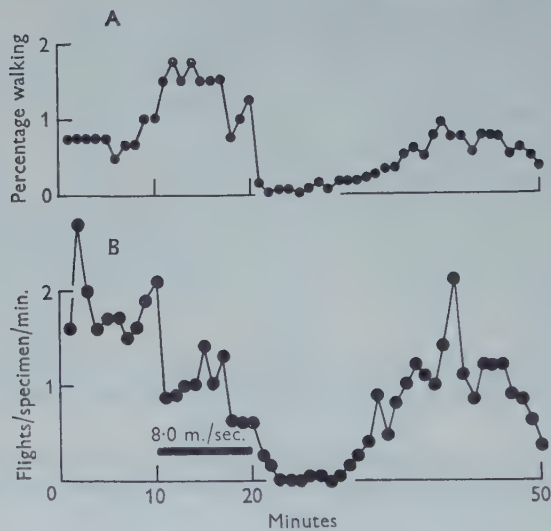


Fig. 12. Flies with antennae immobilized; effect of change of wind speed from 0.5 to 8.0 m./sec. and back. A, Walking activity; B, flight activity.

When these insects with the antennae immobilized were subjected to a wind speed of 8.0 m./sec. for 5 or 10 min., a reduction of flight activity still occurred in five out of ten experiments. Some flight activity was, however, obviously prevented from appearing at 8.0 m./sec. by the mechanical inability of the flies on the down-wind end to make recognizable flights. This mechanical effect was initially rather greater in flies with the antennae immobilized than in normal flies, because in the former an increase of wind from 0.5 to 8.0 m./sec. caused a down-wind movement during the first 5 min., from which there was a recovery (Table 4), complete by 11-15 min. after the change to the higher wind speed. The increase of walking activity during the period at high wind speed (Fig. 12) showed that in these insects activity in fact increased at 8.0 m./sec.

We may therefore conclude that the greater part of the inhibiting effect of the changes of wind speed from 0.5 to 4.5 and to 8.0 m./sec. resulted from movement of the third antennal joint relative to the head.

Activation

In normal flies, so long as they had not previously been rendered insensitive, the stimulus of wind at speeds below 0.5 m./sec. caused activation (Figs. 3-5). The same effect occurred at higher speeds when the antennae had been immobilized with wax and the initial activity of the insect was low. It can be seen from the experiments listed in Table 3 that the upper limit of initial activity for the appearance of activation by the change of wind from 0.5 to 4.5 m./sec. was about 0.5 flight/specimen/min. For this reason the stimulating effect of wind appeared more commonly at low light intensities (Table 3, Exps. 7-12). It also appeared in these operated insects when they were torpid immediately after recovery from anaesthesia or after the halteres had been fixed down with wax. This increase of activity at 4.5 m./sec. in flies with immobilized antennae might be considered to be a spurious effect arising from an increased number losing their footing when relatively torpid. This was shown not to be the case by the fact that the numbers walking also increased at the higher wind speed (Fig. 11).

The absence of activation by a wind of 4.5 m./sec. in these operated flies when the initial activity was above a certain level was comparable to its absence at 0.5 m./sec. in normal flies after prolonged flight or exposure to strong wind. This suggests that when the insects were active their own movements ensured sufficient adaptation to the wind for them not to react to it. The increase of walking amongst the antennae-immobilized flies at 8.0 m./sec was not restricted to groups with an initial low level of activity.

The activating effect of wind was therefore present at all speeds, although in normal insects its appearance in a strong wind was suppressed by the more powerful inhibition.

Experiments were carried out to assess to what extent the large bristles on the head might be responsible for activation.

The structure of the head of *Calliphora* is very similar to that of *Muscina stabulans*; Hollick (1940) found that *Muscina* when attached to a support would carry out flight movements after the antennae had been immobilized but not after the head have been covered with a celloidin capsule. These results suggested that in the latter case receptors other than the antennae may be affected (Chadwick, 1953). The front of the head bears a number of prominent bristles (Fig. 9) and experiments were therefore carried out to see if these might be responsible for the activation. Although the facial bristles and vibrissae move very little, the arista and the frontals and orbitals are all deflected by a slight wind. These latter groups (that is the arista and the frontals and orbitals, together with the single large bristle borne on each second antennal joint) were therefore amputated from a number of specimens of *Calliphora* by clipping them off as close to the base as possible under chloroform-ether anaesthesia, while the flies were held by the wing to avoid damaging the other

bristles left intact. When attached to a support in the wind tunnel, flight movements were still possible in both males and females, although they occurred rather less readily than in normal flies.

When free in the insect chamber, *Calliphora* only became obviously orientated into the wind at speeds of 4.5 m./sec. and above, but it seemed likely that if the sensitivity to wind were strictly localized, the bristles on the head might yet be those mainly concerned. In a series of experiments, these same bristles were amputated from groups of twenty to forty flies which were then tested for the effect of the change of wind speed on flight and walking activity, each experiment consisting of three repeated changes of wind speed from 0.5 to 0.05 m./sec. Each experiment was accompanied by a control using flies which had previously been anaesthetized but not operated upon.

Six out of eight experiments following removal of these bristles still showed activation by the higher wind speed. The flies used for these were comparable to those in the other experiments described in this paper in that they had been moderately active for some days before these observations. In the remaining two experiments the activating effect was abolished and inhibition took its place. These two experiments were carried out with flies which had been kept under quiet conditions in the breeding cages for 2 or 3 weeks prior to the experiments. Although insufficient to define closely the conditions under which the head bristles are important, these experiments sufficed to show that while these bristles may sometimes contribute to the activating effect of wind, they were not the only organs or mechanism concerned.

The reduction of activity following exposure to strong wind

When a population of normal flies was subjected to an increase of wind from 0.5 m./sec. to a higher value, activity was immediately depressed and then increased as the flies adapted to the new conditions. In a certain number of cases this increase of activity was not maintained and activity declined again after a while. In all cases, however, the resumption of the low wind led to the rebound to a high level of activity as in Figs. 1 and 2.

When the same experiment was carried out with flies in which inhibition was suppressed by immobilization of the antennae, the depressing effect of a wind of 8.0 m./sec. was more obvious, setting in from the first few minutes of the period at high wind speed. Return to low wind speed resulted in a further immediate fall in flight and walking activity, followed by a slow rise (Fig. 12). The occurrence of these effects is set out in Table 5.

A family of curves for the subsequent depression of activity at 0.5 m./sec. in flies with the antennae immobilized is given in Fig. 13, flight activity being expressed as a percentage of that at 0.5 m./sec. before the period of high wind speed. These curves show that the depression was greater and lasted longer following longer periods at 8.0 m./sec.

The post-inhibitory rebound to high activity shown by normal flies was therefore a dominant rebound superimposed upon an activity curve of this nature, arising from some other effect of the wind. The fact that normal flies and those with the antennae

Table 5. Occurrence of reduction of activity after prolonged exposure to high wind speed in normal flies and in flies with antennae immobilized

	Wind speed (m./sec.)	No. of experiments in which winds maintained at high value for 20 min. or more	No. of experiments in which depressing effect of wind begins during period at high wind	Activity shown when 0.5 m./sec. wind speed resumed
Normal flies	8 6 5 4.5 2	4 1 1 16 1	2 } (depressing effect sets in 10-20 min. after onset of high wind) 0 1 4 1	Rebound to high level of activity
Flies with antennae immobilized	8	4 Exps. with winds at high value for 15 min. or more	4 (effect sets in during first few min.)	Further fall in activity (9 expts.)

waxed but not immobilized became very active after exposure to a high wind showed that flight was not limited by the wind having caused undue fatigue or injury. It seems likely that the low activity was largely a rebound from excessive activation by the high wind. The fact, however, that the rebound was so large compared with the apparent magnitude of the activating effect, and that the depression of activity set in during the period at high wind speed, suggests that additional factors may have been involved.

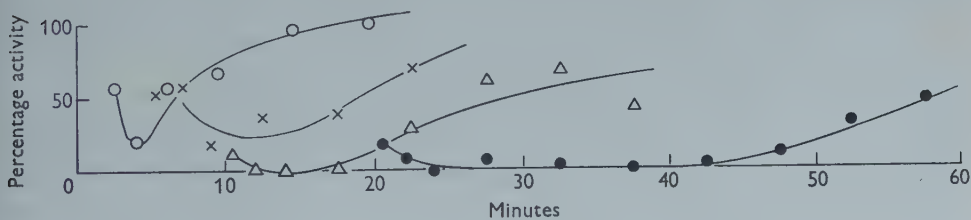


Fig. 13. Flies with antennae immobilized; recovery curves at 0.5 m./sec. following varied periods at 8.0 m./sec.

DISCUSSION

The effect of change of wind speed

The experiments of Davies (1936) and Haine (1955 *a, b*) have shown that wind causes inhibition of flight in aphids. No laboratory investigations so far appear to indicate that wind increases the numbers of insects taking to flight, although stimulation by wind has been shown to play a part in the initiation and maintenance of flight in *Muscina* (Hollick, 1940) and in locusts (Weis-Fogh, 1949, 1956). The present results have shown that wind can cause an increase or a decrease of activity in *Calliphora* according to the range of wind speed, the optimum being at about

0.7 m./sec. under the particular experimental conditions. The differences in adaptive changes in activity following changes of wind at lower and at higher speeds provide an interesting contrast between a reaction in which inhibition is not important, and one in which it is dominant. In the low wind speed range from still air to 0.5 m./sec. in which wind has an activating effect and inhibition is very slight, a decrease of wind causes an immediate decrease of activity without subsequent recovery, and an increase causes a more or less gradual increase of activity. The state of low activity tends to persist as in recovery from low light intensity (Digby, 1958). In the higher wind speed range where inhibition is involved, both increase and decrease of wind speed cause a sharp response which is followed by a considerable degree of adaptive recovery over a period of 20 min. or more.

The nature of the reactions to wind

It has been shown that the effect of wind speed is complex. There is an activating effect from the lowest speeds to the highest; there is an over-riding inhibitory effect with the threshold at about 0.7 m./sec.; and there is a further depressing effect of prolonged high wind. It has been possible to show that under some conditions the bristles of the head account for a large part of the activating effect, but in previously active flies their effect is negligible. It has also been possible to show that the inhibiting effect of strong wind arises from movement of the third antennal joint.

The extent to which the activation and inhibition of walking and flight activity may be related to the reflex mechanisms of initiation and maintenance of flight (see Pringle, 1957) is uncertain, although it seems likely that there may be some parallel. For instance, the small activating effect of wind on the bristles of the head is comparable to its effect on flight activity in the mounted insect.

Conditions are not, however, strictly comparable in the two cases. Thus when flies are at rest on a surface a strong wind has an activating effect which is normally suppressed by the inhibiting action of the antennae; it does not necessarily follow that the activating component occurs in insects in which the air-flow arises by virtue of their own flight, because it may well arise in some way from the stresses imparted by the insects clinging to the surface. Movement of the third antennal joint of *Muscina* was shown by Hollick (1940) to affect the path of the wing-tip during flight and to cause the reflex lifting of the legs when a jet of air was directed at the head. These reflexes are of obvious importance in flight, but it is difficult to see what part the inhibiting effect of movement of the third antennal joint of *Calliphora*, described in the present paper, might play in flight itself. During flight in a mounted insect the third joint vibrates rapidly, and it seems probable that it may then produce an even more powerful inhibiting stimulus than in an insect at rest. If so, it may perhaps serve to balance in some way the activating stimuli arising from other sources during flight. It would follow that after each flight a normal fly would experience a rebound to a state of higher excitation than would a fly with the antennae immobilized. That such may be the case is supported by the

fact that immobilizing the antennae reduced the number of flights compared with those made by normal controls, although the actual flights made by the insects with immobilized antennae were apparently normal.

Ecological significance

Lack of sufficiently detailed field studies on the reactions of *Calliphora* or other dipterous flies to wind speed so far precludes comparison of these laboratory experiments with field behaviour. These experiments do, however, suggest the type of behaviour which is likely to occur in the field. Under the experimental conditions activity increased with wind speed up to about 0.7 m./sec. and decreased with increase of wind above this speed. The optimum speed probably varies with many factors, but nevertheless the experimental figure of about 0.7 m./sec. is above the range of wind speed usually found under the microclimatic conditions experienced by an insect at rest. So far as this optimum prevails in the field an increase of wind speed alone may be expected to increase activity in sheltered situations, in vegetation and close to the ground, and to decrease it in exposed situations. The activating effect of a low wind speed in the field is likely to be much greater than is indicated in these experiments, as the insects are probably adapted to still air conditions to a much greater degree, and this would enhance the effect. Wind in the field is normally turbulent, and in more exposed situations where speeds above the optimum prevail the inhibition of activity by wind would tend to result in reduced activity during gusts and increased activity during lulls. The occurrence of a rebound from the inhibition by wind would tend to allow a maximum amount of activity during windy weather for a minimum amount of displacement of the population by wind.

It is to be noted that under sunny conditions in the field the activating and inhibiting effect of wind is likely to be complicated by the results of substantial changes of temperature arising from heat loss by convection, variation of which is particularly great at the low speeds prevailing under microclimatic conditions (Digby, 1955). The effect of wind by itself is to be sought under overcast conditions.

Field observations of locusts (Strelnikov, 1936; Kennedy, 1939; Waloff & Rainey, 1951; Rainey *et al.* 1957) have suggested that very similar reactions may occur in these insects. Thus the first downward movements made by roosting locusts early in the morning appear to be partly stimulated by light gusts of wind which therefore have an activating effect. These insects have been quiescent during the night; later in the day when they become more active flight is inhibited by moderate winds and under gusty conditions they take to flight during the lulls and land during the gusts. In strong winds they remain on the ground and walk up-wind. Although there are great differences between the structure and ecology of *Calliphora* and these locusts, it is interesting that the field behaviour of the locusts in these respects agrees with that to be expected of *Calliphora* from the experiments described in this paper.

SUMMARY

1. A study has been made of the effect of wind speed on the flight activity of a population of the blowfly *Calliphora erythrocephala* in a small wind tunnel.
2. Under the experimental conditions wind has an activating effect up to about 0.7 m./sec., above which it inhibits flight.
3. Below 0.5 m./sec., activity decreases rapidly with decrease of wind speed and then remains at or close to the lower value. The increase of activity arising from increase of wind speed is much slower, the state of low activity tending to persist.
4. At speeds between 0.5 and 4.5 m./sec. the changes of activity in response to both increase and decrease of wind are very rapid and are followed by a considerable degree of adaptation in each case.
5. Inhibition of activity by the change of wind speed from 0.5 to 4.5 m./sec. and to 8.0 m./sec. results from the movement of the third antennal joint relative to the head.
6. When this antennal movement is prevented, the change to 4.5 m./sec. causes activation if the initial activity is low, and the change to 8.0 m./sec. causes an increase of walking activity although flight is decreased for mechanical reasons.
7. Exposure to the higher speeds causes an additional depression of activity not dependent on movement of the third antennal joint.

I am indebted to Dr A. G. Hamilton for facilities for this work, and to Professor Sir Alister Hardy, F.R.S., in whose department initial experiments were carried out under a grant from the Agricultural Research Council. I am grateful to Prof. D. V. Davies for his careful criticism of the manuscript and to my wife for her assistance in preparing this paper.

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WAVE PROPAGATION ALONG FLAGELLA

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The nature of the mechanism which initiates and propagates transverse bending waves along the length of a flagellum has been the subject of discussion for many years. Two possible mechanisms have been advocated: (i) that the flagellum is a passive elastic filament set in motion by an active process located at its proximal end, the propagation of the wave along the filament being the result of its elastic properties; (ii) that actively contractile elements exist along the length of the flagellum. The latter view has received the greatest support, but no definite proof appears to have been established.

In this paper the problem is approached theoretically by considering the types of wave propagation which can occur along an elastic filament immersed in a viscous medium.

To simplify the analysis the following assumptions have been made: (i) the flagellum is circular in cross-section; (ii) the waves are propagated in a plane; (iii) inertial forces are negligible; (iv) the amplitude of the waves is sufficiently small* that only the forces normal to the axis need be considered.

Assumptions (i) and (ii) are not inconsistent with the biological evidence (Gray, 1955).

At the very low Reynolds number appropriate to flagellar motion (of the order of 10^{-3} – 10^{-5}) assumption (iii) is certainly valid. Although assumption (iv) is not true for observed flagellar motion, results derived using it are not likely to be greatly in error even when applied to large-amplitude waves.

THE MODES OF WAVE PROPAGATION ALONG A FLAGELLUM

Two sets of forces govern the motion of a flagellum and hence determine the form and rate of propagation of waves along it: (i) elastic forces which tend to straighten the flagellum; (ii) viscous forces which oppose the motion of each element through the water.

Consider the forces acting on an element dx of the flagellum (Fig. 1).

The elastic force dF_E is given by equation (1), which occurs in the theory of vibrating beams (Morse, 1948):

$$dF_E = -QSK^2 \frac{\partial^4 y}{\partial x^4} dx, \quad (1)$$

where Q is Young's modulus, S is the area of cross-section and K is the radius of gyration of the section.

* For clarity, wave amplitudes in all diagrams have been shown large.

The viscous force dF_V can be calculated from the formula for the drag of a cylinder at low Reynolds numbers (Lamb, 1932):

$$dF_V = \frac{4\pi\mu}{2.0 - \log R} \frac{\partial y}{\partial t} dx, \quad (2)$$

where μ is the viscosity and R is the Reynolds number.

Strictly this relation is non-linear, since R is a function of $\partial y/\partial t$. At these very low Reynolds numbers, however, the coefficient in (2) varies only slowly with R , and may, without serious error, be considered constant over most of the cycle of oscillatory movement. Only as the velocity approaches zero does this approximation break down, but here both dF_E and dF_V are approaching zero, so little error will result.

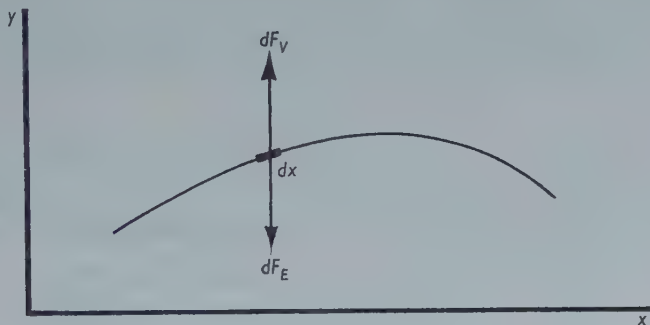


Fig. 1. Forces acting on an element of a flagellum.

Since dF_E must equal dF_V ,

$$-QSK^2 \frac{\partial^4 y}{\partial x^4} dx = \frac{4\pi\mu}{2.0 - \log R} \frac{\partial y}{\partial t} dx, \quad (3)$$

or

$$\frac{\partial^4 y}{\partial x^4} = -\frac{1}{QSK^2} \frac{4\pi\mu}{2.0 - \log R} \frac{\partial y}{\partial t}. \quad (4)$$

The steady-state solution of this differential equation can be shown to be

$$y = e^{i2\pi ft} (Ae^{r_1 x/l_0} + Be^{r_2 x/l_0} + Ce^{r_3 x/l_0} + De^{r_4 x/l_0}), \quad (5)$$

where f is the frequency of oscillation, r_1, r_2, r_3, r_4 are the four values of $\sqrt[4]{-i}$; A, B, C, D are complex constants depending on the conditions at the two ends of the flagellum and l_0 is the 'scale length' given by

$$l_0 = \sqrt[4]{\left(\frac{QSK^2}{2\pi f} \cdot \frac{2.0 - \log R}{4\pi\mu} \right)}. \quad (6)$$

Each of the four terms of equation (5) represents one of the four types of wave (or 'modes') which can propagate along the elastic filament (Fig. 2). The relative amplitude and phase of the modes depend on the conditions at the ends of the

filament. The characteristics of the four modes are given in Table 1, where the 'damping distance' is the distance the wave travels before its amplitude falls to $1/e$ of its original value.

On an infinite unconstrained flagellum only the principal mode will exist. This wave carries the flux of energy distally along the flagellum, the continuous loss of energy along the length manifesting itself as a decrease in amplitude. Any lateral constraint (e.g. a rigid hinge) at the proximal end will initiate the secondary mode. If, in addition, the flagellum is of finite length, both principal and secondary modes are reflected at the distal end to give modes III and IV.

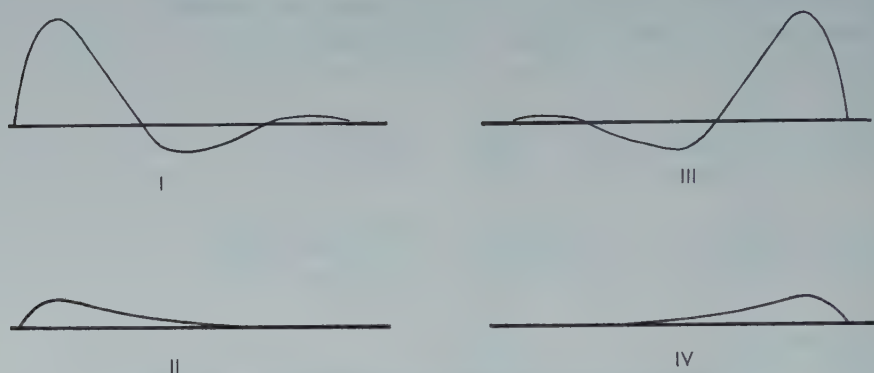


Fig. 2. Modes of wave propagation along a flagellum.

It is important to note that all wavelengths and damping distances are proportional to l_0 , the scale length. This length depends on the frequency of oscillation, the mechanical properties of the flagellum and the nature of the medium; it is the only term in which these parameters enter into the wave equation. It thus follows that the *form* of the waves propagated along a flagellum is independent of all these parameters. Variation of stiffness, frequency, viscosity, etc., only affect the *scale* of the waves.

Table 1

Designation of mode	Direction of propagation	Direction in which amplitude decreases	Wave-length	Damping distance
I. Principal	Increasing x	Increasing x	$6.8l_0$	$2.6l_0$
II. Secondary	Decreasing x	Increasing x	$16.5l_0$	$1.1l_0$
III. Reflected principal	Decreasing x	Decreasing x	$6.8l_0$	$2.6l_0$
IV. Reflected secondary	Increasing x	Decreasing x	$16.5l_0$	$1.1l_0$

As mentioned earlier, the relative amplitudes of each of the four modes are determined by end conditions. In the next section one specific set of proximal end conditions will be imposed and the resultant wave-patterns computed.

THE FLAGELLUM DRIVEN BY ANGULAR OSCILLATION
OF THE PROXIMAL END

If the proximal end of a filament is constrained to move about a rigid hinge, and the energy is fed in by angular displacement of the constrained end, the system approximates to a flagellum attached to a stationary head. The boundary conditions are then:

$$y=0 \quad \text{at} \quad x=0 \quad \text{for all } t, \quad (7)$$

$$\frac{\partial y}{\partial x} = G e^{i2\pi ft} \quad \text{at} \quad x=0. \quad (8)$$

Here G determines the amplitude of the motion.

At the distal end, both bending moment and shearing force vanish:

$$\frac{\partial^2 y}{\partial x^2} = 0, \quad (9)$$

$$\frac{\partial^3 y}{\partial x^3} = 0. \quad (10)$$

If the total length of the flagellum is L , equations (5) and (7)–(10) give

$$y = G l_0 e^{i(2\pi ft + \pi/4)} \left\{ \frac{1}{2 + 2 \cos \phi \cosh \phi} \right\} \times \{ \sin \theta + \sinh \theta + \sin \phi \cosh (\phi - \theta) - \cos \phi \sinh (\phi - \theta) - \cosh \phi \sin (\phi - \theta) + \sinh \phi \cos (\phi - \theta) \}, \quad (11)$$

where $\theta = \frac{x}{l_0} (0.92 - 0.38i) \quad (12)$

and $\phi = \frac{L}{l_0} (0.92 - 0.38i) \quad (13)$

Wave-patterns have been computed from equation (11) for various values of L ; the results are shown in Fig. 3. Since distances are expressed in terms of l_0 , these patterns will represent the motion of any passive flagellum, whatever the values of the physical parameters. In Fig. 3 the shape of the flagellum at successive intervals of one-eighth of a complete cycle is displayed; the dotted line represents the envelope of the motion.

DISCUSSION OF THE CALCULATED FLAGELLAR MOTIONS

The motion of the infinite flagellum constrained at its proximal end will be considered first. Under such conditions the maximum amplitude occurs at about $1.2 l_0$ from the proximal end (Fig. 3 H). From this point onwards the principal wave predominates; its amplitude decays exponentially, and is virtually zero at $10 l_0$. Irrespective of its length, the flagellum can exhibit only about $1\frac{1}{2}$ visible wavelengths.

When the flagellum is of finite length the reflected waves affect the resulting wave-pattern, the amplitude of the distal end increasing with progressive shortening of the length. When $L = 3 l_0$ the amplitude of the free distal end is greater than that of any other part of the flagellum; the envelope acquires a characteristic fish-like form

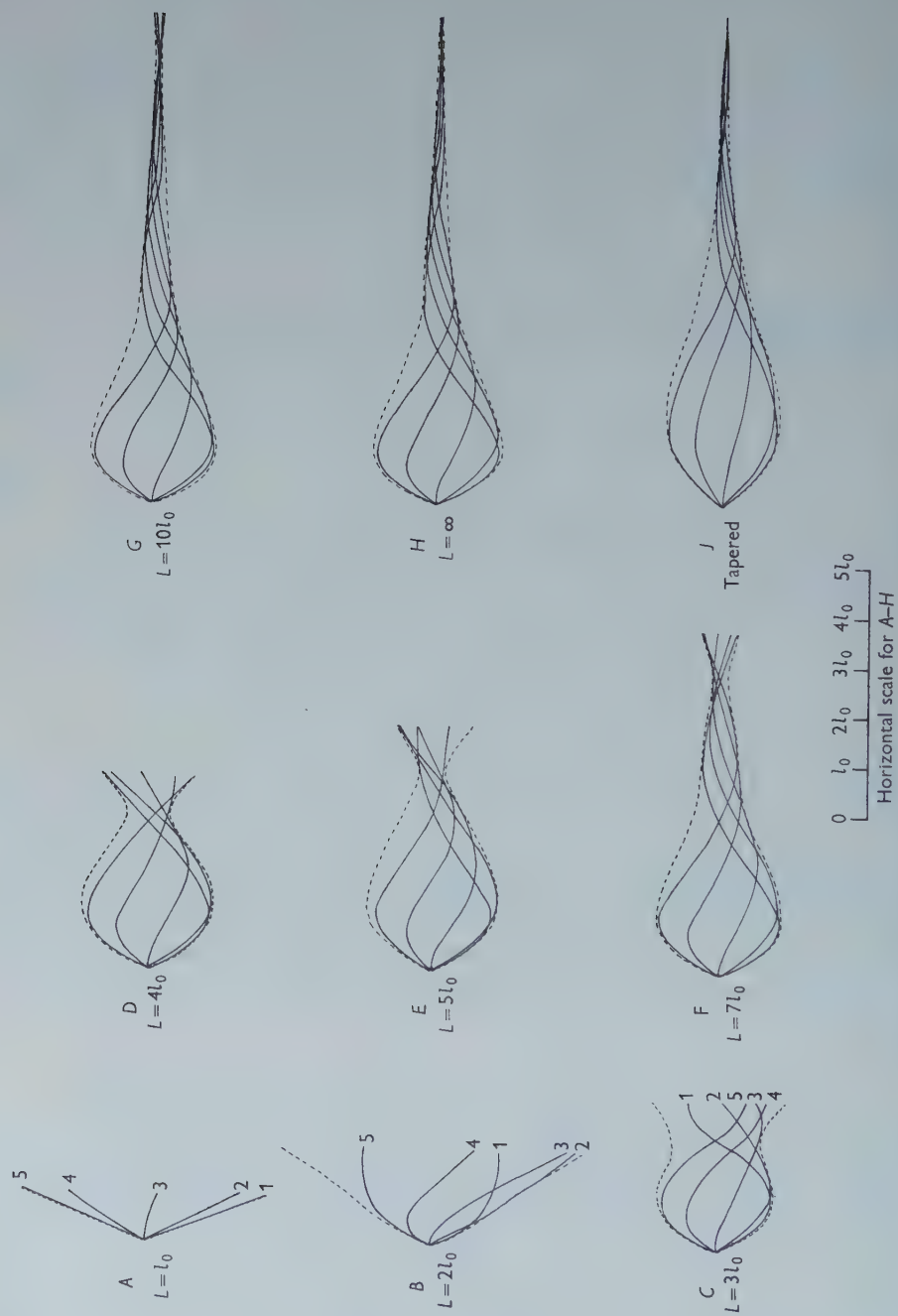


Fig. 3. Calculated wave-patterns on a flagellum. Vertical amplitudes have been exaggerated for clarity.

but contains less than one wavelength. When $L < 2l_0$ the motion approximates to that of a relatively rigid cilium.

Two of the arguments purporting to show that distributed contractile elements must exist along flagella are: (i) Since in observed flagellar motions the point of maximum amplitude occurs some way from the proximal end, energy is being fed in near this point; (ii) inertial forces are necessary to maintain a progressive wave system. The results of Fig. 3, derived for a weightless passive flagellum, show that these arguments are not valid.

However, it is clear from Fig. 3 that a passive elastic flagellum of uniform cross-section driven from one end cannot exhibit more than $1\frac{1}{2}$ wavelengths along its length. Further, the amplitude of the wave decreases exponentially. If a flagellum exhibits more than $1\frac{1}{2}$ wavelengths, or has a sustained amplitude along its length, the propagation of the waves cannot be due to a passive mechanism. This conclusion is unaffected by the nature of the drive at the proximal end, since the secondary wave becomes negligible beyond $3l_0$.

So far only uniform flagella have been considered. The behaviour of a flagellum whose size or mechanical properties vary along its length can readily be described, provided that the variation is slow compared with the wavelength. Under these conditions the value of l_0 , the scale length, will vary along the flagellum, giving a non-linear 'stretch' to the wave-patterns of Fig. 3. The result of this is shown in Fig. 3J drawn for a flagellum whose distal end is one-third of the diameter of the proximal end. Again waves of sustained amplitude appear impossible.

So far as is known, the only instance in which the envelope of an actual flagellum has the form of any of the curves of Fig. 3 is in the case of 'ageing' spermatozoa described by Gray (1955). On the other hand, passive models described by Gray (1955) have envelopes very similar to those of Fig. 3.

It appears, then, that under certain abnormal circumstances sperm tails behave as though they were passive, but that to explain their normal motion an active mechanism is essential.

BENDING MOMENTS IN AN ACTIVE FLAGELLUM

As it seems clear that the waves exhibited by a passive elastic filament cannot resemble those observed on a normal flagellum, it is of interest to consider the alternative hypothesis of actively bending elements distributed along the length of the filament.

If a distribution $B(x)$ of active bending moment along the flagellum is postulated, equation (1) becomes

$$dF_E = -QSK^2 \frac{\partial^4 y}{\partial x^4} dx - \frac{\partial^2 B}{\partial x^2} dx, \quad (14)$$

and (3) becomes

$$-QSK^2 \frac{\partial^4 y}{\partial x^4} dx - \frac{\partial^2 B}{\partial x^2} dx = \frac{4\pi\mu}{2.0 - \log R} \frac{\partial y}{\partial t} dx; \quad (15)$$

therefore

$$\frac{\partial^2 B}{\partial x^2} = -QSK^2 \frac{\partial^4 y}{\partial x^4} - \frac{4\pi\mu}{2.0 - \log R} \frac{\partial y}{\partial t}. \quad (16)$$

This equation gives implicitly the form of $B(x)$ corresponding to any specified motion.

As an example the form of $B(x)$ will be calculated for the case of a sine wave of constant amplitude b and wavelength λ propagating along the flagellum.

Putting
$$y = b e^{i2\pi(ft-x/\lambda)}, \quad (17)$$

$$QSK^2 = \alpha \quad (18)$$

and

$$\frac{4\pi\mu}{2.0 - \log R} = \beta, \quad (19)$$

equation (16) gives

$$-\frac{1}{b} \frac{\partial^2 B}{\partial x^2} = \alpha \left(\frac{2\pi}{\lambda} \right)^4 e^{i2\pi(ft-x/\lambda)} + i2\pi f\beta e^{i2\pi(ft-x/\lambda)}, \quad (20)$$

or

$$B = b e^{i2\pi(ft-x/\lambda)} \left\{ \alpha \left(\frac{2\pi}{\lambda} \right)^2 + i2\pi f\beta \left(\frac{\lambda}{2\pi} \right)^2 \right\}. \quad (21)$$

This represents a progressive wave of bending moment with a wavelength, velocity and direction of propagation similar to the wave of displacement; there is, however, a phase difference between the waves.

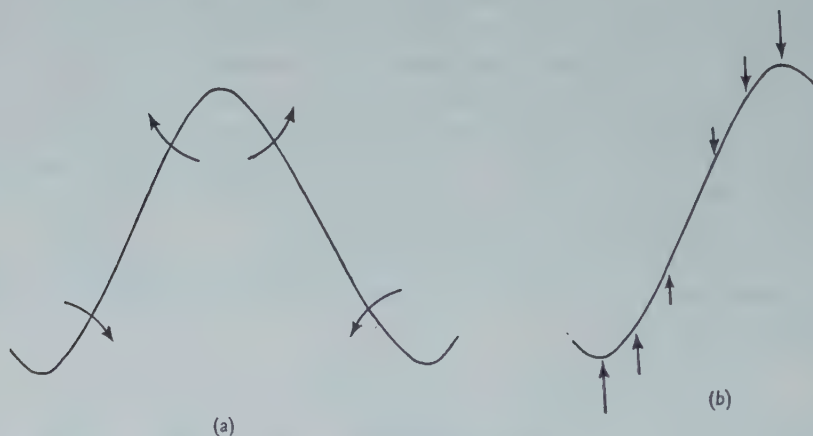


Fig. 4. Forces on a flagellum.

Equation (21) comprises two components, corresponding to the two terms within the brackets. The first component is a progressive wave in phase with the wave of displacement and represents the bending moment necessary to produce the sinusoidal elastic deformation of the filament. The second component is a progressive wave in quadrature with the wave of displacement, being the bending moment required to equalize the viscous forces on the filament. The origin of these two components is illustrated in Fig. 4. In (a) the elastic forces tending to straighten the filament are shown; it is clear that the maximum bending moment occurs at the point of maximum displacement. The viscous forces shown in (b) produce a maximum moment at the point where the displacement is zero.

The resultant total bending moment is the sum of the two components; if elastic forces predominate, the resultant is nearly in phase with the displacement, while if viscous forces predominate, the resultant approaches quadrature with the displacement.

ENERGY CONSIDERATIONS FOR AN ACTIVE FLAGELLUM

Some of the energy fed into each element of the flagellum will be used to overcome viscous forces; the remainder is absorbed in elastic deformation of the element. Unless a reversible mechanism exists to convert this elastic energy back into chemical energy, it is irrecoverable and will degrade into heat in the elastic or contractile components. While there is some evidence for reversibility in vertebrate muscle under special circumstances (Abbott, Aubert & Hill, 1951) it seems unlikely that in the present case any appreciable fraction of the elastic energy will be recovered.

On this assumption, the power expended in the flagellum can be shown to be

$$P = b^2 L \left\{ \frac{\beta}{2} (2\pi f)^2 + \alpha f \left(\frac{2\pi}{\lambda} \right)^4 \right\}. \quad (22)$$

In this equation the first term represents the power used in overcoming viscous forces, and the second the power required for the elastic deformation.

Taylor (1952) and Hancock (1953) have shown that for a small undulating organism carrying waves of small amplitude the forward velocity v is given by

$$v = \frac{2\pi^2 b^2 f}{\lambda}. \quad (23)$$

From (22) and (23)

$$v = \frac{P/L}{(2\pi/\lambda)^3 \alpha / \pi + \beta (2\pi f) (\lambda/2\pi)}. \quad (24)$$

For a given power P and frequency f , equation (24) gives a maximum value of v when

$$\left(\frac{\lambda}{2\pi} \right)^4 = \frac{3\alpha}{2\pi^2 f \beta}. \quad (25)$$

Substituting from equations (6), (18) and (19)

$$\lambda = 6.3 l_0.$$

Comparison of this value with those given in Table 1 shows that for an active flagellum the maximum propulsive efficiency (i.e. maximum forward speed for a given power) is attained when the wavelength of the actively generated wave is very nearly the same as that of the principal wave on a passive flagellum.

At the condition for maximum efficiency it can be shown from equation (22) that one-quarter of the power is used for elastic deformation, the remainder being dissipated by viscous forces. Furthermore, from equation (21) it can be shown that the real and imaginary components of the wave of bending moment are very nearly

equal. This indicates that the progressive wave of bending moment leads the wave of displacement by about one-eighth of a complete cycle.

For a wavelength other than the optimum, the phase difference between the waves of bending moment and displacement will lie between 0 and $\frac{1}{2}\pi$.

THE ACTIVATION OF CONTRACTILE ELEMENTS

A flagellum can be idealized into an elastic filament surrounded by or enclosing a series of bilaterally arranged contractile elements, differential contraction of elements on the two sides producing localized bending moments (Fig. 5). The relation between the tension and length of the elements and the displacement and bending moment of the flagellum is given in Table 2, where *A* and *B* refer to the elements on the opposite sides (Fig. 5).

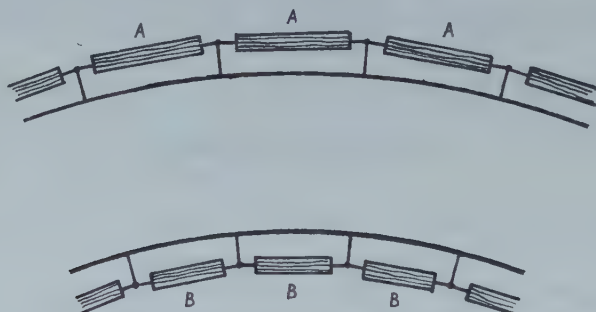


Fig. 5. Contractile elements surrounding an elastic filament.

Table 2

<i>A</i>		<i>B</i>	
Tension	Bending moment	Tension	Bending moment
High Low	Negative Positive	High Low	Positive Negative
Length	Displacement	Length	Displacement
Long Short	Positive Negative	Long Short	Negative Positive

It was shown earlier that for maximum propulsive efficiency the wave of active bending moment leads the wave of displacement by about $\frac{1}{4}\pi$. From Table 2 it will be seen that for both elements *A* and *B* this implies that the tension should lag on the length by about $\frac{3}{4}\pi$. The phase relations for the two elements are shown in Fig. 6.

Pringle (1957) has suggested that the rhythmic activity of a spermatozoon might arise from a myogenic mechanism; this suggestion will now be examined in the light of the present results. In studies of insect fibrillar muscle, Boettiger, Machin & Pringle (1958) have shown that these muscles behave like a combination of two components: (i) an ordinary visco-elastic element and (ii) an elastic element in

which the force due to a change of length only appears after a time delay of the order of a few milliseconds. In the muscles studied, the ordinary elasticity was rather larger than the 'delayed elasticity'; for sinusoidal movements a phase lag of only about $\frac{1}{4}\pi$ between length and force could occur. It is of course somewhat speculative to relate this result to the minute contractile elements of a flagellum. It seems, however, not unreasonable to postulate that such a delayed elastic effect could predominate in these contractile elements; a phase lag of $\frac{3}{4}\pi$ between force and length might then be sustained.

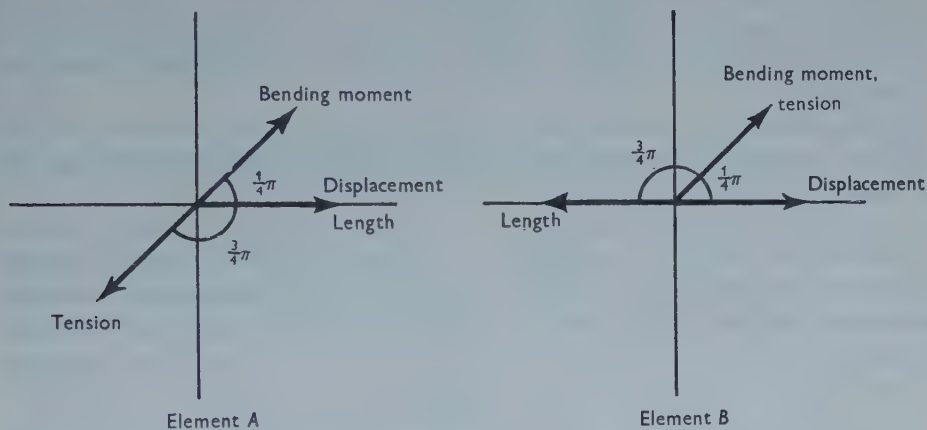


Fig. 6. Phase relationships for contractile elements.

In this way actively induced bending at the proximal end of a flagellum could give rise to a propagated wave whose amplitude was sustained by energy arising in the more distal elements. These elements would be activated when deformed by a passively propagated wave of bending from the immediately proximal region. No mechanism for the transmission of control information to the contractile elements would be necessary.

Such a system could account for the following observed phenomena: (i) a flagellum is motionless when separated from the middle piece of the spermatozoon or from the basal granule (Gray, 1955); (ii) a small obstruction causes the more distal region of the flagellum to become motionless (Gray, 1955)—further experiments on this effect would be valuable; (iii) while fresh spermatazoa show sustained amplitude along all their length, ageing sperms have an envelope similar to those drawn in Fig. 3 for passive filaments (Gray, 1955). It is postulated that as the sperms age the contractile elements in the tail become inactive, although the oscillatory bending at the proximal end continues.

QUANTITATIVE COMPARISON WITH OBSERVATION

As already shown, for maximum propulsive efficiency the wavelength on an active flagellum must be of the order of $6l_0$. The value of l_0 , the scale length, is given by equation (6). Using the parameters given by Gray (1955) and Gray & Hancock (1955)

for the spermatozoa of *Psammechinus miliaris* the value of l_0 is 1.8μ , corresponding to a wavelength for maximum efficiency of 12μ ; the observed value is 24μ . In view of the approximate nature of the theory and of the value of Young's modulus employed, the prediction of the wavelength to within a factor of two is satisfactory.

SUMMARY

1. The types of bending waves which can propagate along a thin elastic filament immersed in a viscous medium are derived.
2. It is not possible to account for the form of the waves observed on actual flagella if the flagellum is regarded merely as a passive elastic filament driven from its proximal end.
3. The observed wave forms can be explained by assuming active contractile elements distributed along the length of the flagellum. These elements could be activated by local bending.

I wish to thank Prof. Sir James Gray for introducing me to the problem, and for his continued help and advice. I am also indebted to Lord Rothschild for valuable criticism and to Elisabeth Machin for assistance with the mathematical aspects of the work.

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THE MECHANISM OF GILL VENTILATION IN THREE FRESHWATER TELEOSTS

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(Received 3 April 1958)

(With Plate 13)

INTRODUCTION

The general nature of the respiratory movements of teleost fishes has been known for a long time, but several significant details of the mechanism are only now becoming clear as a result of modern experimental methods. Reviews of the extensive literature have been written by Babak (1921), Leiner (1938), and Fry (1957). Du Verney (1701) is generally credited with the first description of breathing movements in fish, and his view that mouth and opercula expand and contract together was later supported by Paul Bert (1870). However, Bert's method, using ampoules in the buccal and opercular cavities, interfered considerably with the movements. This was pointed out by François-Franck (1906 *a, b*), who was one of several workers to make use of smoked drum techniques. It became clear from this work that movement of the mouth preceded that of the opercula by a brief interval of time in all phases of the breathing cycle. The importance of both the buccal and the opercular valves in producing a unidirectional flow of water was clearly appreciated by Bert. Baglioni (1907, 1910) made a comparative study of different marine fishes and drew up a classification based on the degree of development of the branchiostegal apparatus. Despite some variations between different fishes, his account agrees substantially with that of François-Franck and forms the basis of the descriptions given in most elementary text-books.

These experimental investigations tended to draw attention to the intermittent nature of the water flow into the buccal cavity and out of the opercular cavities. The breathing mechanism was regarded as a single pump because the gill arches were thought to hang quite separately in the buccal cavity, there being no contact between the filaments of adjacent arches. The direction of water flow in such a system would have been parallel to the long axis of the gill filaments, with little movement of water between the secondary lamellae, which are the plates running across the upper and lower surfaces of the filaments.

A completely different view was expounded by Woskoboinikoff (1932), who maintained that the gills were not such passive features of the system but that, by virtue of their arrangement in the living animal, they functionally separated the buccal from the opercular cavities. The gill filaments of adjacent gill arches were

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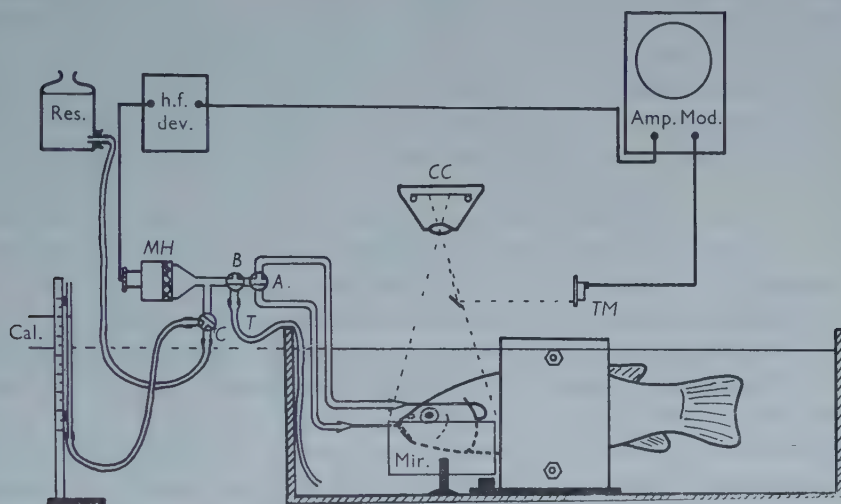
thought by him to interdigitate so that the whole gill structure formed a continuous sieve. The only pathway for the water through this sieve would therefore be at right angles to the long axis of the gill filaments through the small spaces between the secondary lamellae. Van Dam (1938) subscribed to a very similar view of the gill arrangement, and this was finally confirmed by Hofdijk Enklaar (in Bijtel, 1949), who looked at gills directly through a celluloid window inserted in the operculum and saw that the tips of the filaments remained in contact throughout the normal breathing cycle. In Woskoboinikoff's scheme it was proposed that the gills also acted as a valve. He stressed the importance of the phase of opercular abduction during which the pressure was lowered in the opercular cavity, water being drawn through the gills by the action of this so-called opercular suction pump. During opercular adduction the gills in some way prevented reflux of water, the opercular valve opened and the water was forced out of the cavity. Woskoboinikoff ascribed little function to the buccal apparatus in this paper.

In subsequent papers, however, Woskoboinikoff & Balabai (1936, 1937) corrected this over-emphasis on the suction pump and suggested that the water current was maintained by the action of a buccal force pump before the gills and of a suction pump behind them. These important papers have not been widely known and indeed were unknown to the authors when the major part of the work in this paper was carried out. Nor were they mentioned by van Dam (1938) who was the first worker to consider in any detail the nature of the flow of water across the respiratory epithelium. He drew attention to two important functional adaptations which are involved in the high utilization (50–80%) of oxygen that he measured. The first of these is that the direction of water flow is opposite to that of the blood circulation in the secondary lamellae. Secondly, he suggested that the flow of water across the respiratory surfaces is continuous throughout the whole breathing cycle. He substantiated this suggestion by connecting the two cavities externally by means of a glass tube and showing that water in this tube always moved in the direction mouth-to-operculum. From this experiment van Dam concluded that the pressure in the buccal cavity was always greater than that in the opercular cavity. These views were incorporated in a generalized scheme of teleostean breathing mechanisms by Henschel (1939).

Measurements of the pressures in the two cavities had in fact been made by Woskoboinikoff & Balabai at about the same time as van Dam was doing his work, and in general their observations confirmed the hypothesis outlined above. However, the pressure in both cavities fluctuated and they found that, while both the maximum and minimum values in the buccal cavity were generally higher than the corresponding pressures in the opercular cavity, there was an overlap in the two ranges such that the maximum pressure in the opercular cavity was often greater than the minimum pressure in the buccal cavity. In order to obtain further proof of van Dam's suggestion of a continuous flow of water across the gills it is therefore important to know the exact time course of these pressure fluctuations. Observations of this kind have been reported briefly (Hughes & Shelton, 1957) and more detailed descriptions are given in the present paper.

METHODS

Measurement of pressures, such as those produced by the respiratory movements of fish, presents considerable practical difficulties, particularly if a study is to be made of their time course. The manometer should have a high natural period in order to record rapid changes of pressure. In addition, the connexions between the respiratory chambers and the manometer must be made with fine tubing so that the breathing movements are not restricted in any way. In all manometers these two features are in some degree mutually exclusive and in water manometers, such as those used by Woskoboinikoff & Balabai in their quantitative measurements and by van Dam in his qualitative demonstration, an adequate compromise cannot be obtained.



Text-fig. 1. Diagram of the apparatus used for the simultaneous recording of movements and pressures. A detailed description will be found in the text.

In the present experiments a condenser manometer (Hansen, 1949) consisting of a manometer head (*MH*, Text-fig. 1) and 'electrical aggregate' (the high-frequency device (*h.f. dev.*) and d.c. amplifier (*Amp.*)) was used. The connexions between the buccal and opercular cavities and the manometer were made with short lengths of hypodermic tubing of 0.5 mm. outside diameter joined to stainless steel tubing of much larger diameter and bore. In the first series of experiments only one such tube was connected to the manometer, and to change the connexion from one cavity to the other involved exchanging the hypodermic tubing and readjusting its position. Subsequently a three-way tap (*A*) was obtained to which two large diameter connecting tubes, made of lead, were attached (Text-fig. 1). This made it possible to insert the fine hypodermic tubes into both cavities at the beginning of an experiment. Because the hypodermic tubes entered the cavities through the mouth and opercular opening they were of necessity arranged along the line of water flow. To avoid dynamic complications in the pressures recorded, a small hole was

made in the side of the tubing near its tip and the hole at the tip was sealed. Thus the connexion between the pressure source and the recording apparatus was at right angles to the main direction of water flow. Connected to the manometer head by another tap (*C*) was the reservoir (Res.) and the calibrator (Cal.), the latter providing a head of water of known height above the level of water in the fish tank. The zero pressure level on the oscilloscope screen was set when the manometer head was connected directly to the water in the tank by tube *T*. When the manometer was working under these conditions a full-scale deflexion on the oscilloscope screen could be produced when a pressure of 1 cm. of water was applied to the manometer head, the noise level being equivalent to 0.5 mm. of water. The natural frequency of the instrument when a pressure was applied suddenly at the open end of the hypodermic tubing was about 100 cycles/sec.

Cinematography was used to record the breathing movements, the photographs being taken at speeds of 16–30 frames/sec. with either a Sinclair 35 mm. or a Zeiss 16 mm. ciné camera. The camera (*CC*, Text-fig. 1) was arranged to photograph the dorsal view of the fish's head directly and the side view reflected in a mirror (*Mir.*) inclined at 45°. Illumination was provided by two photofloods and two spotlights. Subsequent analysis of the ciné films enabled accurate plots to be made of respiratory movements which had not been hampered by mechanical restraints. The two points from which plots of the mouth and opercular movements were obtained are shown in Pl. 13, which is a single frame from one of the ciné films. The positions of the buccal and opercular valves could also be plotted. A time marker (*TM*, Text-fig. 1; Pl. 13) with a rotating contact was included in the camera field, and this made it possible to match exactly the pressure records and movement plots (Hughes, 1958).

When these experiments were done only one Hansen manometer was available, and so successive recordings of the pressures within the buccal and opercular cavities were taken. The two recordings were made within a minute of each other. In the regularly breathing fish these records were readily superimposed by using the movement plots obtained at the same time as the pressure records. Another method used in superimposing successive pressure traces, particularly when no ciné films were taken, was to display the opercular movements as deflexions of the second beam on the cathode-ray tube. An RCA 5734 transducer valve was used for this, the movements being transmitted to the anode pin of the valve by a long lever arm resting lightly on the operculum.

Three species of fish were examined by these methods, the tench (*Tinca tinca*), the roach (*Leuciscus rutilus*) and the trout (*Salmo trutta* f. *fario*). Some recordings were also made of the breathing movements and pressures in the pike (*Esox lucius*), but this fish was not examined in detail as the results were similar to those obtained from the trout and roach. The tench and roach used were between 45 and 70 g. in weight and the trout were a little larger, the heaviest weighing approximately 80 g.

The fish were deeply anaesthetized in a 0.5% urethane solution and placed in a clamp, which firmly held the trunk and head (Shelton, 1958). The clamp was then

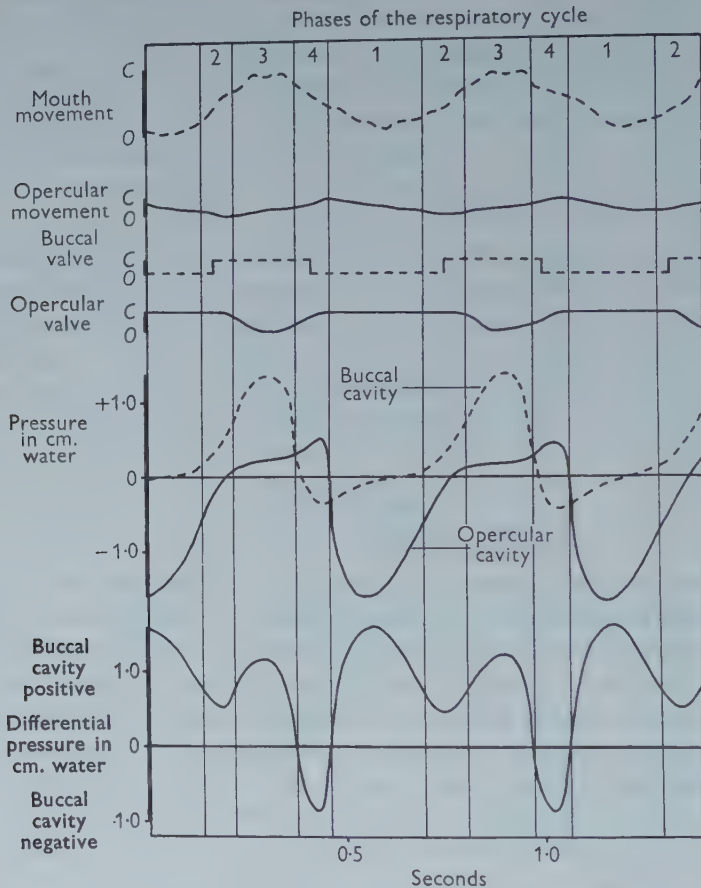
secured in the experimental tank which was of 10 l. capacity. After the fish had been arranged in the apparatus, the urethane concentration was reduced to approximately 0.2%, which allowed the fish to recover to a lightly anaesthetized level sufficient to keep it quiet during the experiment. This solution was constantly aerated and its temperature maintained at 17°–20° C. The difficulties involved in working on respiratory processes in fish are well known (Fry, 1957). Activity or excitement cause wide fluctuations in metabolic rate and in the general breathing activity. Anaesthesia overcomes some of these difficulties, particularly when it is intended to experiment on the breathing mechanisms or metabolic rate of the inactive fish. However, it must be recognized that anaesthesia may introduce its own complications, and largely because of this possibility it has not found favour as a means of suppressing excitement. Urethane in high concentrations produces breathing which is slower and deeper than normal. In still higher concentrations the respiratory movements get smaller and less frequent until eventually they cease. At low levels of anaesthesia such as those used here, however, there seems to be little effect on the respiratory patterns or responses, though the animal remains quiet. These therefore were the conditions under which the experimental results were obtained.

RESULTS

A. The respiratory cycle in the trout

The respiratory rhythm is extremely regular and in the trout used in these experiments there were usually 100 cyc./min. A typical result obtained by superimposing pressure curves and movement plots of the mouth and operculum can be seen in Text-fig. 2. In all the figures and descriptions the pressure at the water-level in the experimental tank will be called zero pressure, so that the pressures higher and lower than this become positive and negative respectively. All the determinations were made relative to this zero level, and descriptions are greatly facilitated if this convention is retained. As Fig. 2 shows, the durations of the opening and closing phases were approximately equal for both the mouth and the opercular movements but movements of the mouth preceded those of the operculum by one-fifth to one-quarter of a cycle. As changes in volume of the buccal and opercular cavities accompanied these movements it was to be expected that similar relationships would also apply to the pressure curves. Closing of the mouth and operculum is associated with an increase in the pressure within the buccal and opercular cavities respectively. The pressure in both cavities exceeds that of the surrounding water during this part of the cycle; this positive pressure is greater in the buccal cavity. Correspondingly, as the mouth and operculum open the cavities increase in volume and the pressures within them become negative with respect to the surrounding water, but in this case the pressure change in the opercular cavity is the greater. Thus during these two main phases of the cycle the pressure in the buccal cavity is positive with respect to that in the opercular cavity, as is shown by the differential pressure curve. This generalization is not true for the whole cycle, however, because there is a brief period during which the pressure in the buccal cavity is

negative with respect to that in the opercular cavity. But in general the pressure curves confirm earlier suggestions (Woskoboinikoff & Balabai, 1936, 1937; van Dam, 1938; Henschel, 1939) that the flow of water across the gills is maintained by



Text-fig. 2. *Trout* (70 g.). The breathing movements of the mouth and operculum, together with associated pressure changes in the buccal and opercular cavities. The differential pressure between these cavities is shown below. O and C indicate the opened and closed positions of the mouth, operculum and their associated valves. Temp. 17° C.

a dual mechanism. The first component of this involves a buccal pressure pump which forces water through the gills and the second component, the opercular suction pump, draws water through the gills into the opercular cavity. These two main phases are separated by two periods of transition, so that a complete breathing cycle may be artificially divided into the following four phases.

(1) *The opercular suction pump predominant*

The negative pressure produced in the buccal cavity, as this increases in volume, gradually falls as the mouth reaches the fully open position and then begins to

close. At this time the operculum is abducting and as the opercular cavity is closed externally by the opercular valve a considerable negative pressure is produced and so water flows through the gills from the buccal cavity.

(2) *Transition with a fall in the differential pressure between the buccal and opercular cavities*

The mouth continues to close and the buccal cavity decreases in volume. At first water leaves the buccal cavity through the still open mouth and the increase in pressure within the cavity is small. By the time the closing movement is about one-third of the way to completion, closure of the buccal valves diminishes this reflux and the pressure within the buccal cavity begins to rise more steeply. Meanwhile, the pressure in the opercular cavity is becoming less negative because water is still flowing through the gills into the maximally expanded opercular cavity, the operculum having almost reached the end of its abduction stroke. The opercular valve opens just as the pressure in the opercular cavity becomes equal to that in the external medium.

(3) *The buccal pressure pump predominant*

The operculum now begins to adduct, and during this initial period of adduction there is a considerable gap between the operculum and the flank of the animal. There is little resistance to the movement of water from the cavity to the exterior and so the positive pressure produced in the cavity is small at first. The positive pressure in the buccal cavity, on the other hand, rises steeply to its maximum value as the mouth closes and then begins to fall off as the final stages of the closing movement are reached. Water is therefore forced over the gills during this phase and there is little reflux through the mouth as the upper and lower lips are quite close together, making the buccal valves much more effective.

(4) *Transition with a reversal of differential pressure*

At this point, however, the mouth begins to open, the volume of the buccal cavity increases and the buccal valves open. As the aperture produced by the opening mouth is small at first, the pressure within the buccal cavity falls rapidly, reaching a maximum negative value when the mouth is half open. The operculum, on the other hand, continues to adduct and the pressure within the opercular cavity becomes increasingly positive, no doubt because the space through which water from the cavity can escape to the outside is becoming smaller. Finally, a point is reached when the gap between operculum and flank becomes vanishingly small so that water flow from the cavity must be negligible. Though the pressure is still positive within the opercular cavity, this is the point at which the opercular valve appears to close on many of our records (see below). As the pressure within the buccal cavity is negative with respect to that in the opercular cavity, there will be a tendency for the flow of water across the gills to be reversed during this phase of the movement cycle.

(5) *The opercular suction pump predominant*

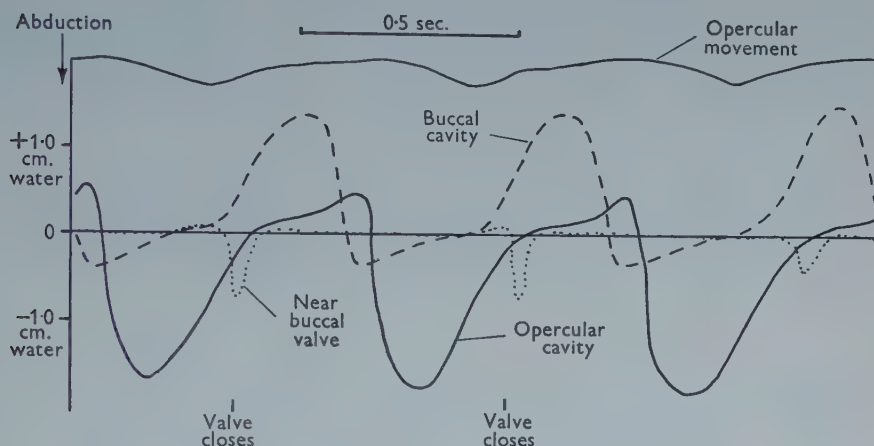
The operculum begins to abduct and as the valve is closed the pressure within the cavity falls extremely rapidly to its maximum negative value. Any reversal of water flow will no doubt contribute initially to the sudden nature of this pressure change. The process then continues with the next cycle.

By dividing the respiratory cycle into these four phases stress is laid upon the flow of water across the gills rather than on its flow into the buccal cavity and out of the opercular cavity. Such a division has much to recommend it in a system whose main characteristic is a unidirectional flow of the respiratory medium in contrast to the tidal flow characterizing those systems which are usually divided into expiratory and inspiratory phases.

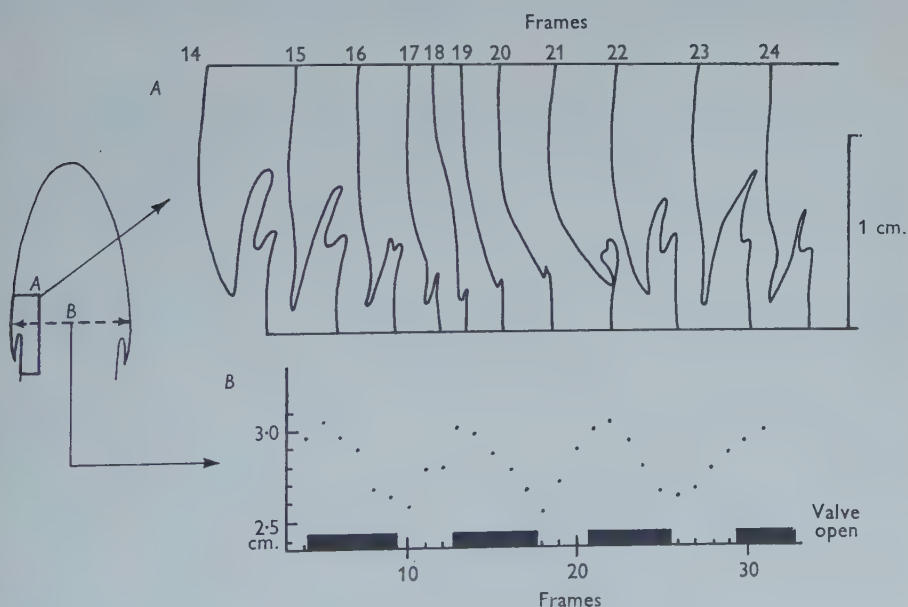
This can be regarded as the typical respiratory cycle. In the trout it is relatively constant in its many components, but even in this species we have observed differences between individuals and, at different times, in the same individual. For example, the differential pressure may be reduced because the positive pressure in the opercular cavity and/or the negative pressure in the buccal cavity are relatively greater than those shown in Text-fig. 2. The presence of a transition phase during which the opercular pressure is greater than the buccal pressure has invariably been found. One of the most difficult parts of the analysis has been the timing of the buccal and opercular valves. The general picture, obtained from analyses of both trout and tench breathing, suggests that these act as passive valves, since they tend to open and close according to the direction of the pressure gradient.

The action of the buccal valve was studied from films taken with specially arranged illumination. In addition, it was found that if the pressure was recorded just inside the buccal cavity near the maxillary valve a characteristic small negative pressure occurred at the instant when the valve closed, as confirmed by comparison with the films. The nature of this pressure is obscure, but it made it possible to decide when the valve closed without the necessity of filming. The result of superimposing pressure records obtained from the buccal and opercular cavities with such a record is shown in Text-fig. 3. It was possible to study the action of the opercular valve in films taken with the standard illumination, since its posterior edge was clearly silhouetted against the floor of the tank. Text-fig. 4 shows the outline of the operculum and its valve throughout the cycle. The degree of expansion of the operculum is indicated, together with the position of the valve. The point at which the valve opens is easily established and can be seen just to precede the position of maximum abduction of the operculum. The instant at which the valve is truly closed is more difficult to decide, as the tracings show. Although the valve appears to be closed at frame 17-18 it is quite possible that it is not really closed until frame 19. This is of particular interest, as a number of our analyses suggest that the valve closes when the pressure within the opercular cavity is positive with respect to the surrounding water. Though this appears improbable if the valve is a passive one, it is possible if, during the later stages of its adduction, the operculum is actively

pressed against the side of the body, as it were short-circuiting the valve. The appearance of the valve on the ciné records would agree with this interpretation.



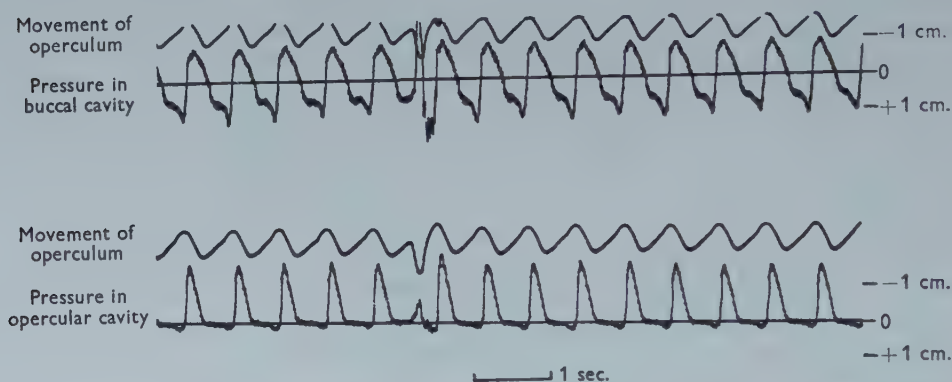
Text-fig. 3. *Trout*. Superimposed pressure records from the buccal and opercular cavities, and in the neighbourhood of the buccal valve. The instant at which the valve was observed to close on a ciné film taken simultaneously is indicated.



Text-fig. 4. *Trout*. (A) The outline of the left operculum and its valve seen in dorsal view traced from successive frames of a ciné film (20 frames/sec.). (B) The opercular movements obtained by measuring the distance across the fish at this level are plotted below, together with the time during which the valve appeared to be open.

B. *The respiratory cycle in the roach*

The frequency of the movements was again about 100 cyc./min. This rhythm was occasionally interrupted by 'coughs' which were rarely found in the trout. This behaviour has been described by several authors (Bijtel, 1949; François-Franck, 1906*a*) and the function usually ascribed to it is that of cleaning the gills. The pressure changes produced in the course of one type of 'cough' can be seen in Text-fig. 5. During one of these interruptions the operculum, though making

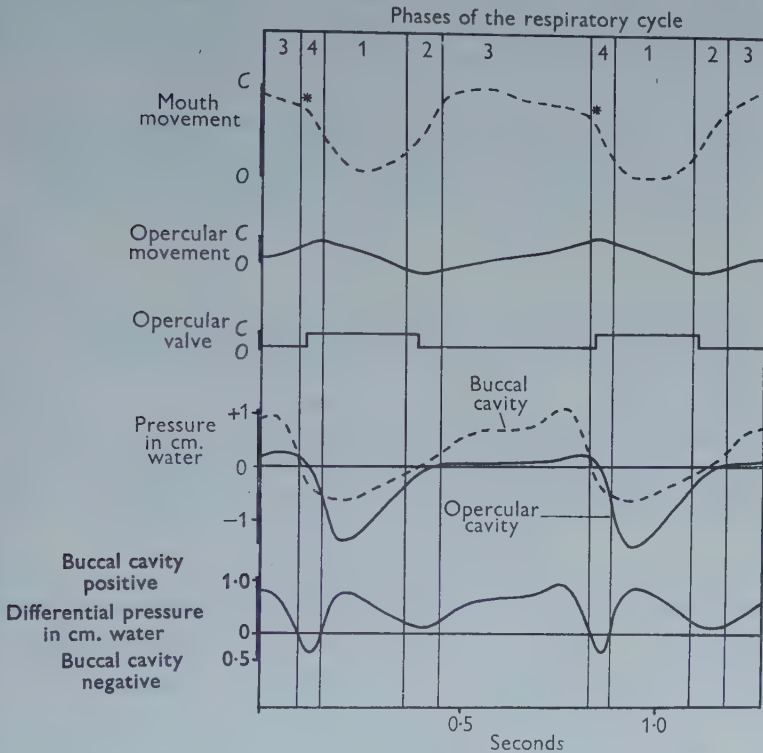


Text-fig. 5. Pressure curves recorded from the buccal and opercular cavities in the roach. A single 'cough' is included on each record. Transducer records of the opercular movements are shown on the second beam of the oscilloscope, abduction of the operculum being downwards in all records.

some small movements, does not close sufficiently to obstruct the opening into the opercular cavity and the mouth opens and closes rapidly. This means that there is relatively little change in pressure in the opercular cavity whilst there are two large changes, first negative and then positive, in the buccal cavity. These fluctuations must cause the water flow through the gills to be reversed and then to be re-established in the original direction. Sudden changes in the direction of water flow of this sort, together with movements of the gill filaments themselves (Bijtel, 1949), are effective in cleaning the respiratory surfaces.

The pattern of normal breathing is extremely similar to that described in detail for the trout, and the respiratory cycle can be divided into the same four phases. Superimposed pressure curves and movement plots are shown in Text-fig. 6. The mouth begins to open about one-eighth, and to close about one-fifth, of a cycle before the corresponding movements of the operculum. The period during which the mouth opens and the operculum abducts occupies less than half a cycle so that the wave-form of the movement plots is not so regular as that in the figure of trout breathing. In the roach, after the mouth has closed completely, rotation of the mandible and maxillae causes the lips to move backwards relative to the rest of the head. The volume of the buccal cavity continues to decrease during this time and a positive pressure is still recorded therein. Unfortunately this backward move-

ment causes those points on the upper and lower jaws, which are used in plotting movements, to move apart. Thus the mouth appears to be opening gradually in Text-fig. 6 when it is in fact closed. The instant when the mouth begins to open is indicated by an asterisk. The upper and lower lips then move forward and a negative pressure is produced within the buccal cavity by the lateral and dorso-ventral expansion of its walls. After a delay period, during which the pressure in



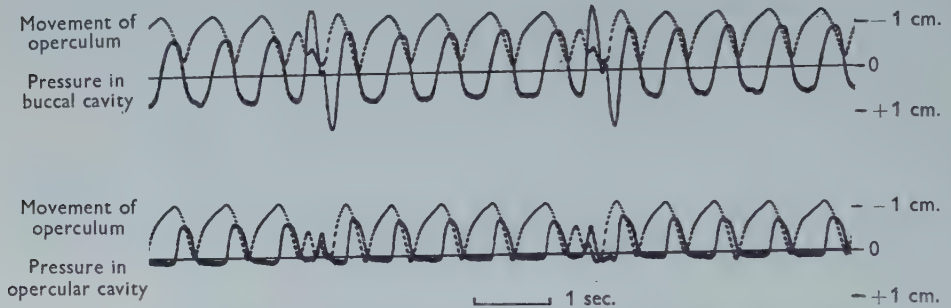
Text-fig. 6. *Roach* (60 g.). The breathing movements of the mouth and operculum, together with associated pressure changes in the buccal and opercular cavities. The differential pressure between these cavities is shown below. O and C indicate the open and closed positions of the mouth, operculum and their associated valves. Temp. 18° C.

the opercular cavity is positive with respect to that in the buccal cavity, the operculum begins to abduct and the negative pressure associated with this movement is produced. The opercular valve is closed during this phase but it is very difficult to be certain of its precise movements. The valves in the individuals we examined were shorter than those in the tench and trout. They appeared to remain open longer along the dorsal border than on the mid-opercular border and so the movements were not so readily plotted from the films. However, the movements of the mid-opercular border of the valve have been plotted and appear in Text-fig. 6. They again follow the direction of the pressure gradient. Buccal valves are not obvious in this fish; no doubt the behaviour already described, in which a

considerable part of the buccal pressure pump phase is accomplished with the mouth closed, compensates in some degree for this lack.

C. *The respiratory cycle in the tench*

The exact superposition of pressure curves and movement plots was difficult because of irregularities in the breathing rhythm of the tench. There are usually about 60 breathing cycles per minute but they are regularly punctuated by 'coughs' which are more frequent than in the roach. The pressure changes produced during a 'cough' (Text-fig. 7) are often very like those seen in the roach and are caused by

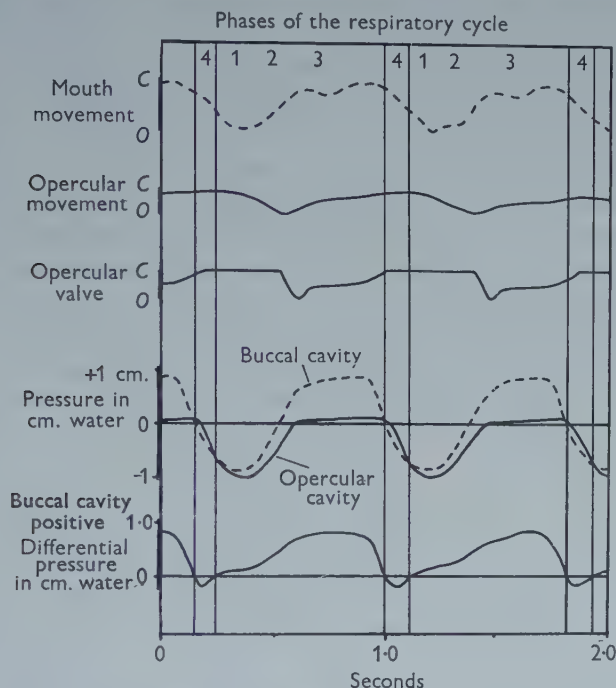


Text-fig. 7. Pressure curves recorded from the buccal and opercular cavities in the tench. Two 'coughs' are visible on each record. Transducer records of the opercular movements are shown on the second beam of the oscilloscope, abduction of the operculum being downwards in all records.

the same pattern of movement. The cycles both before and after a 'cough' often vary both in amplitude and frequency and so each 'cough' has a considerable effect on the rhythm. Because of this, and because of the greater range of individual variation, many more experiments were carried out on this species. When the records could be superimposed satisfactorily results such as those in Text-fig. 8 were obtained. During all phases the mouth movements precede those of the operculum by about one-eighth to one-fifth of a cycle. Once again the backward rotation of the upper and lower jaws when the mouth is closed forms a distinct part of the cycle. This can be seen in the mouth movement plots of Text-fig. 8 as a second hump during the closing movement. As in the roach this results in the final stages of the buccal pressure pump phase (phase 3) occurring when the mouth is closed. When the mouth begins to open, the pressure in the buccal cavity falls rapidly and there is the usual momentary reversal in pressure gradient between the two cavities. Abduction of the operculum then follows and the pressure within the opercular cavity becomes negative. The most striking feature of the pressure curves, however, is the relatively large negative pressure which is recorded in the buccal cavity during this phase. In some experiments it exceeds that in the opercular cavity (Text-fig. 7). In the majority of records the negative pressure in the buccal cavity, though large, is not so large as that in the opercular cavity. In these cases, as in the trout and roach, the pressure in the buccal cavity is positive with respect

to that in the opercular cavity, with the single exception of phase 4 of the breathing cycle.

The action of the opercular valve is readily visible in the film. Its movements, in general, follow the pressure gradient between the opercular cavity and the external environment, yet some analyses show it to be open when the pressure inside the opercular cavity is negative (Text-fig. 8). In the tench from which the



Text-fig. 8. *Tench* (60 g.). The breathing movements of the mouth and operculum, together with associated pressure changes in the buccal and opercular cavities. The differential pressure between these cavities is shown below. O and C indicate the opened and closed positions of the mouth, operculum and their associated valves. Temp. 18° C.

records of this figure were obtained, it was observed that methylene blue pipetted near the posterior border of the operculum entered the cavity momentarily, as the valve opened, and was then expelled. An active valve with its own musculature could produce this sort of effect, but this is not the only possible explanation. As the operculum abducts the valve has to bridge a gap, which is increasing in size, between the opercular flap itself and the side of the body. A point must be reached when the valve, because of its dimensions or stiffness, is pulled away from the flank. If the operculum continues to abduct after this has occurred then water will enter the opercular cavity from outside. It is difficult to be absolutely certain that this is the correct interpretation because the valve moves from the closed to the maximally open position in one twenty-fifth of a second and so the whole procedure is recorded on, at the most, two frames of the ciné film. This was by no means a constant feature of the breathing pattern of the tench, however, and in other

individuals there was no inflow of water to the opercular cavity as the valve opened. Analyses confirmed that the pressure in the cavity was positive when the valve opened in these fish.

In order to decide what sort of range existed in breathing behaviour a group of six fresh tench were treated in an identical way as regards depth of anaesthesia, time intervals before pressure records were made, etc. The mouth or the opercular movements were recorded by means of the transducer valve. The results confirmed that the pattern of breathing as shown in Text-fig. 8 is the most common. In all six fish, and indeed in every tench examined, a very distinct positive pressure was developed in the buccal cavity, always exceeding that produced in the opercular cavity. The main variable was the relative size of the negative pressures recorded in the two cavities. In three of the tench the negative pressure in the buccal cavity was distinctly less than that in the opercular cavity. In the other three fish there was very little difference between the pressures in the two cavities during phases 1 and 2. In occasional records from two of the fish the negative pressure in the buccal cavity during these phases was greater than that in the opercular cavity.

In view of these variations it was interesting to find later that Woskoboinikoff & Balabai (1937) also record similar variations from the basic pattern in this fish. They found, for instance, that in 22 % of the cases the negative pressure in the opercular cavity was less than that in the buccal cavity and that in 9 % they were approximately equal.

DISCUSSION

The use of cinematography has made it possible to record and analyse the breathing movements with as little interference with the fish as possible. This method has the added advantage that the action of the buccal and opercular valves could be observed at the same time. The recording of pressure changes simultaneously with the filming of movement provides a more complete picture of the functioning of this complex system than has previously been possible. The results obtained support those of François-Franck and others on the time relations of the movements and have confirmed the relationships expected between these movements and the pressure changes in the buccal and opercular cavities. Because of the differences between the pressure curves the system must be considered as consisting of three chambers, the opercular cavities being separated from the buccal cavity by a continuous gill sieve offering resistance to water flow. It is not a single cavity in which the gills are freely suspended.

The differential pressure curves (Text-figs. 2, 6 and 8), obtained by subtraction of the opercular from the buccal pressures, show two maxima in each cycle. The opercular suction pump and the buccal pressure pump alternately produce these pressure maxima and, during the transitions between their action, periods of very low-pressure differential occur. Indeed, as the suction pump takes over from the pressure pump, the differential becomes negative and the flow will tend to reverse. Division of the ventilating system into these two pumps is justifiable, provided it is realized that the terms refer only to the passage of water across the gills. The

opercular suction pump is not directly involved in drawing water into the system nor the buccal pressure pump in forcing it out.

The relationships of the pressure curves from the buccal and opercular cavities suggest that, except for the very brief period of phase 4, water will flow continuously across the gill filaments from buccal to opercular cavity. It is not possible, however, to make exact deductions about the rate of flow without some knowledge of the properties and numerical value of the gill resistance. The volume of water flowing over the gills at any time is, of course, directly related to the pressure difference which exists between the two cavities. If, to take the simplest case, the resistance were constant and the same for flow in both directions, then the flow would always be related to the pressure difference by the same factor and the form of a curve showing flow across the gills would be identical with that of the differential pressure curve. In order to get some idea of the relationship between differential pressure and flow, simultaneous determinations were made of the flow and of the pressures in the buccal and opercular cavities of the tench. The volume of water passing over the gills was measured by separating the mouth and opercular regions of the fish with a rubber membrane. Any water which came over a spillway in the 'opercular' part of the tank was pumped by the fish from the 'mouth' compartment. The water in the latter was maintained at a constant level which was the same as the level of the spillway in the 'opercular' compartment. A convenient way of varying the rate at which water was pumped through the system was by changing the tension of carbon dioxide in the water by arbitrary amounts. If the gill resistance remains constant the ratio mean pressure/minute volume should be the same for all rates of flow. As Table 1 shows, this is not the case, the ratio decreasing as the minute volume and mean pressure difference increase. It is clear, then, that under these conditions the gill resistance is variable, and that it probably decreases as the pressure difference across the gills increases. Some support for this conclusion comes from preliminary measurements made on fish which had stopped breathing under very deep anaesthesia. Water could be made to flow over the gills of such fish by raising the level of water in the 'mouth' compartment of a tank similar to that described above. Measurements of the flow, made as this pressure head was maintained at progressively greater values, showed that there was an accompanying decrease in the gill resistance.

Table 1

Minute volume	Frequency/minute	Stroke volume (ml.)	Mean pressure (cm. water)	Mean pressure/Minute volume
39	39	0.99	0.51	0.0131
55	35	1.56	0.76	0.0138
66	48	1.38	0.64	0.0097
67	49	1.37	0.75	0.0112
69	41	1.70	0.66	0.0096
82	43	1.90	0.65	0.0080
124	44	2.83	0.91	0.0074
154	57	2.72	1.12	0.0073
164	56	2.94	1.12	0.0068

The changes in volume of buccal and opercular cavities and in area of the mouth and opercular openings are clearly of fundamental importance in determining the form of the pressure curves recorded in the two cavities. Depending upon the relative size of the cavities and on the extent of their volume change, it is possible for either the buccal pressure pump or the opercular suction pump to become the dominant ventilating mechanism in different species. The pressure curves suggest that these two pumps are fairly well balanced in the trout and roach, whereas in the tench the buccal pump appears to be the more important. It is difficult to be quite certain that this is the case, however, because it must now be recognized that another factor, namely the resistance of the gills to water flow, also has a significant effect on the pressures measured in the two cavities. It has been shown that this resistance changes as the mean differential pressure and the ventilation volume are altered. It must be concluded that changes of gill resistance will also occur during a single respiratory cycle as the differential pressure fluctuates. It is probable that in different species differences will exist not only in the numerical value for the mean gill resistance but also in the range over which the resistance varies with changing pressure.

In spite of these variations the results so far obtained conform sufficiently to the same general plan that it seems reasonable to accept the breathing pattern of a fish such as a trout as representing a general, unspecialized mechanism. Variations on this plan have already been described and it is hoped that further investigations will disclose more fully the range of possible modifications.

SUMMARY

1. A study has been made of the respiratory movements of three species of freshwater fish. The time course of pressure changes in the buccal and opercular cavities was recorded and movements of the mouth and operculum plotted from ciné films taken simultaneously.

2. Opening and closing of the mouth precede respectively abduction and adduction of the operculum by about one-fifth of a cycle.

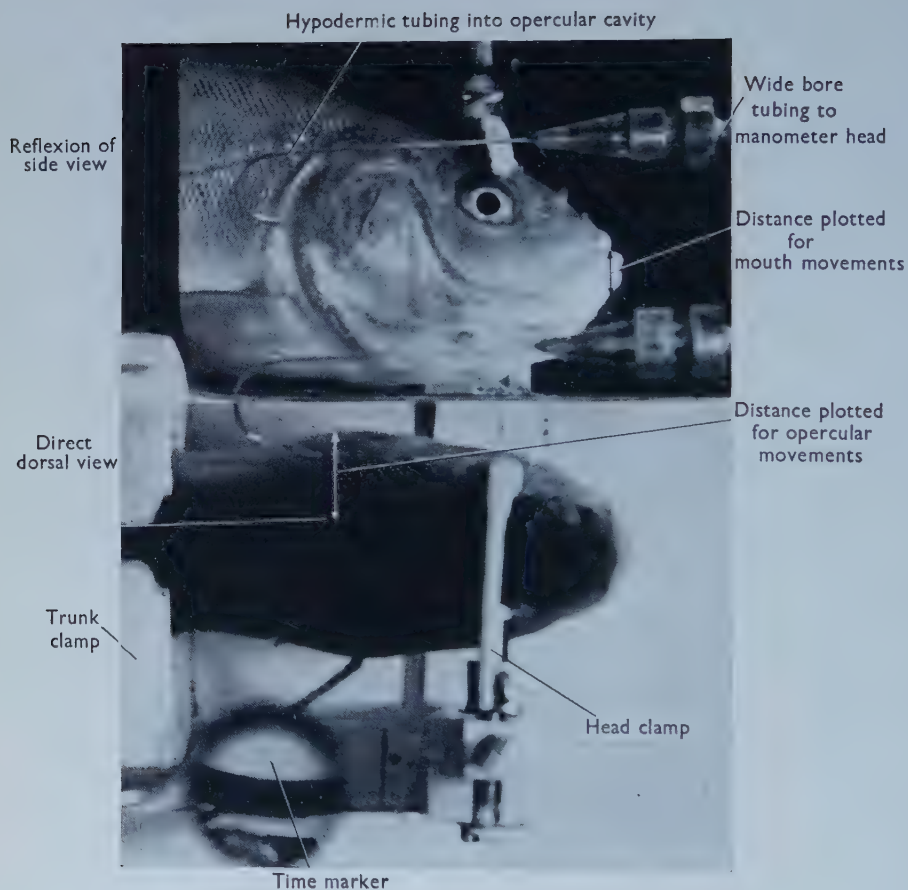
3. The most prominent part of the buccal pressure curve is a positive pressure associated with mouth closing. The size of a negative pressure as the mouth opens is small in the trout but may be relatively large in the tench.

4. Abduction of the operculum produces a marked negative pressure in the opercular cavity of all three species and there is a slight positive pressure during its adduction.

5. The respiratory system is divided into a buccal and two opercular cavities and the concept of gill resistances separating them is introduced.

6. The respiratory cycle is made up of four phases which succeed one another. These are: phase (1) opercular suction pump predominant; phase (2) transition with a reduction in differential pressure between the buccal and opercular cavities; phase (3) buccal pressure pump predominant; and phase (4) transition with reversal of differential pressure.

7. With the exception of phase (4), which occupies only about one-tenth of a



HUGHES AND SHELTON—THE MECHANISM OF GILL VENTILATION IN THREE FRESHWATER TELEOSTS

(Facing p. 823)

cycle, the pressure in the buccal cavity exceeds that in the opercular cavity throughout the cycle. It is therefore concluded that water will flow across the gills for almost the entire cycle but may reverse for this brief period. The quantitative relationship between the pressures and the volume of water flowing across the gills during different parts of the cycle will depend upon the properties of the gill resistances.

We are indebted to several members of the Department of Zoology, Cambridge, for their advice and criticism of this work. One of us (G.M.H.) wishes to thank Dr Tybjaerg Hansen and Mr E. Kaiser of Copenhagen for their generous assistance in the construction of parts of the electric manometer.

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EXPLANATION OF PLATE

A single frame from a ciné film to show a tench set up in the apparatus. The measurements made in plotting the respiratory movements are indicated.

THE EFFECT OF TEMPERATURE CHANGES ON THE THYROID-PITUITARY RELATIONSHIP IN TELEOSTS

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(With Plate 14)

INTRODUCTION

An increase in the thyroid activity of teleosts correlated with an increase in environmental temperature has been recorded by Blanc & Buser (1949), Fortune (1953, 1955), and Barrington & Matty (1954); this has not been confirmed by Delsol & Flatin (1956) who found no effect, or by Olivereau (1950, 1954, 1955*a, b, c*) who demonstrated that increased temperature tended rather to decrease thyroid activity.

The action of the thyrotrophic hormone (TSH) is intensified with increasing temperature (Olivereau, 1955*c*; Delsol & Flatin, 1956). This is confirmed below. Such an action would be expected to lead to a consequent increase in thyroid hormone (TH) production with increased temperature, unless a compensatory decrease in TSH production takes place. Apart from seasonal changes (e.g. Fontaine, 1953; Olivereau, 1954) no information is available on the effect of temperature on the cells of the teleostean anterior pituitary which secrete thyrotrophic hormone (thyrotrophs).

The experiments described here were designed to investigate this aspect and to extend previous observations on the effect of temperature on thyroid activity.

MATERIAL AND METHODS

Phoxinus laevis was used as the main experimental animal; for certain comparative aspects *Carassius auratus* was included.

The fish were kept in groups of six in large glass jars of aerated water. They were fed daily on dried daphnia and the water was changed twice weekly. For chemical thyroidectomy they were immersed in 0.05% thiourea, which was also changed twice weekly. TSH was injected in the form of 'Ambinon' B (Organon Laboratories) which also contains gonadotrophins. 0.05 ml. of Ambinon diluted with Ringer's solution and containing approximately 1-3 Heyl-Laqueur units of thyrotrophin were injected into the body cavity immediately under the pectoral fin. In each experimental series the identical dilution was used. Controls were injected with similar quantities of Ringer solution. The fish were subjected to three environmental temperatures: (a) less than 10° C., (b) 20° C. ($\pm 1^\circ$ C.), (c) 25° C. ($\pm 1^\circ$ C.).

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The experiments were carried out during the period December-early February, and in each specimen both the thyroid and the pituitary were examined.

Thyroid activity was estimated on a histological basis according to the ratio of the area covered by the follicular epithelium to the total follicular area (*e/f* ratio) (Fortune, 1955).

Thyrotrophic activity was also estimated on a histological basis. The specimens were fixed for prolonged periods in Bouin's fluid, which also decalcified them, and the brain and attached pituitary were embedded and sectioned at 5μ in the usual way. Attention was centred on the basophils of the middle glandular region. As this region is small in sagittal section but extends laterally upwards and outwards (Kerr, 1942) only central sections which included the stalk, or those immediately lateral to this region were considered, in order to give comparable pictures of the middle glandular region. The sections were stained with periodic acid Schiff (PAS) method (Pearse, 1953), Gomori's aldehyde-fuchsin (AF) (Dawson, 1953) or Mallory's trichrome. It is possible to distinguish two types of basophil on the basis of their inclusions and staining intensity. One type stains heavily with flocculated inclusions, and cells of this type are regarded as the gonadotrophs; the other type stains less intensely, has finely granular inclusions and a prominent rounded nucleus, and cells of this type are regarded as the thyrotrophs (Kerr, 1942; Atz, 1953; Barrington & Matty, 1955).

Barrington & Matty (1955) found that PAS stained all the basophils whilst AF stained only some of the PAS-positive cells; the latter were experimentally identified as the thyrotrophs. Following the fixation used here this distinction was not valid, and all the basophils stained with both stains (cf. Atz, 1953).

Under certain experimental conditions the staining intensity of the thyrotrophs decreased, accompanied by degranulation and increased vacuolation. This general emptying of the cell was interpreted as indicating increased TSH production. Three grades were recognized: an inactive gland in which the majority of the thyrotrophs showed no marked change in staining intensity or loss of cellular inclusions, an active gland in which the majority of the cells showed pronounced vacuolation and loss of cell contents and an intermediate stage in which at least 50% of the thyrotrophs showed some change indicating emptying of the cells, although this had rarely proceeded as far as the active stage which showed pronounced vacuolation. These are arbitrary grades which merely served to give some measure of comparison between different glands.

EXPERIMENTAL

Thyroid. Effect on thyroid activity of:

(a) '*Ambinon*' injections. *Phoxinus* was acclimatized over a period of 3 days to either 10° C. or 25° C. Twelve fish were used in each experimental group, with twelve controls injected with Ringer and twelve uninjected controls. '*Ambinon*' was injected twice weekly (on opposite sides of the body) and two specimens were killed 24 hr. after each injection. Controls were injected and killed at the same

Table 1. *Effect of 'Ambinon' injections at different temperatures*

<i>e/f</i> ratio after injection	Controls (not injected)		Controls (injected Ringer)		Ambinon injected	
	Temp. 10° C.	Temp. 25° C.	Temp. 10° C.	Temp. 25° C.	Temp. 10° C.	Temp. 25° C.
1	36.5	43.4	41.6	32.6	38.8	50.0
2	33.8	44.0	37.6	35.2	37.0	75.0
3	30.7	36.3	42.8	42.5	44.5	97.9
4	32.5	38.2	32.7	31.2	40.3	89.0
Mean <i>e/f</i>	33.4	39.7	39.2	35.4	40.1	75.5
σ	2.47	3.92	4.51	5.03	3.20	23.7
S.E.	1.24	1.96	2.26	2.52	1.60	11.9

σ is the standard deviation, estimated with one degree of freedom, and S.E. is the standard error of the mean.

intervals. Results of a typical experiment are shown in Table 1. At 25° C. there was an immediate change in the *e/f* ratio after the first injection and this was intensified with further injections. At 10° C. a change in the *e/f* ratio was found after four injections. These results show that the same dose of 'Ambinon' has more effect on the thyroid activity at 25° C. than at 10° C. It was further found that the controls injected with Ringer's fluid tended to show an increase in *e/f* ratio over the uninjected controls. It is also confirmed that the *e/f* ratio of the controls tends to rise with increasing environmental temperature.

(b) *Transference from a high or a low temperature to an intermediate temperature.* In each experiment twelve specimens of *Phoxinus* were placed at room temperature (approximately 17° C.) and over a period of 3 days the temperature was gradually raised to 25° C. A further twelve fish were kept under comparable conditions at 10° C. The fish were maintained at these temperatures for a period of 14 days. Half of each group was then immersed in thiourea and all animals were transferred to 20° C. Specimens were killed and fixed at intervals of 4, 7, 12, and 14 days after the transfer to 20° C. These experiments were repeated through the period December–February, and results of a typical experiment are shown in Table 2.

Table 2. *Effect of previous acclimatization temperature on thyroid activity*

Days	Thiourea-treated		Controls	
	Temp. 25° C.	Temp. 10° C.	Temp. 25° C.	Temp. 10° C.
4	35.2	36.1	38.7	28.9
7	44.0	40.5	32.3	35.0
12	43.4	45.5	36.6	30.9
14	50.0	44.7	30.2	38.7
Mean	43.0	41.7	34.4	33.4
σ	3.16		2.93	
<i>P</i>	0.7		0.8–0.7	

The temperature refers to the pretreatment temperature. σ is the standard deviation of the combined means. *P* is the probability determined using the *t* test.

These results show that there was no significant difference ($P = 0.7$), in the thyroid reaction of fish to the common temperature and that the thyroid activity was effectively the same in both groups. In each group a noticeable, though not pronounced, increase in epithelial height and decrease in the amount of colloid following thiourea treatment had taken place by the end of the period of the experiment.

(c) *Prolonged maintenance under experimental conditions.* Two groups of *Phoxinus* were set up under experimental conditions similar to those in (a). One group was immersed in thiourea. They were kept in the aquarium where the temperature approximated to the outside temperature, for 12 weeks during May–August. The controls for this experiment were the stock fish which were kept in large stone sinks in the aquarium in aerated water and were fed twice weekly on dried daphnia or minced meat. Under this treatment the animals survived in apparently good condition for several months. Fish were killed for examination at weekly intervals after 6 weeks had elapsed. The following values for the e/f ratio were found: control, mean value 45.5 (extremes 31.6–52.2); experimental, mean value 42.8 (26.3–50.1). Those immersed in thiourea showed maximum histological changes after 6 weeks (e/f ratio 90+), and the majority of follicles were completely occluded with greatly heightened epithelium. This was maintained until the 10th week when small amounts of intra-follicular colloid again became visible and the e/f ratio fell to below 90.

Pituitary. Phoxinus. Effect on thyrotroph activity of:

(a) *'Ambinon' injections.* These injections caused a general reduction in the staining intensity of all basophils. There was no appreciable difference in the speed of this reaction at the temperatures employed, but it appeared to be more intense at higher temperatures.

(b) *Variations in environmental temperature.* At temperatures below 10° C. the thyrotrophs remained full and the cell contents stained readily. Only in a few occasional cells were signs of vacuolation and reduction in cell contents noted (Pl. 14A). At 25° C. the thyrotrophs showed some signs of change after a week's exposure to this temperature, and after a fortnight there was a noticeable reduction in the staining intensity and a reduction in the cell inclusions (Pl. 14B). With thiourea at low temperatures some emptying and vacuolation were seen after 14 days' treatment (Pl. 14C). At 25° C. this vacuolation appeared sooner and was more intense, so that after 14 days' treatment the majority of the thyrotrophs were showing all the changes which were interpreted as signs of increased secretion (Pl. 14D).

Table 3. *Effect of temperature in the thyrotrophs*

Treatment	Duration in days	Condition of thyrotrophs
10° C.	14	++
25° C.	14	+-
10° C. + thiourea	14	+-
25° C. + thiourea	14	--

++, inactive grade; +-, intermediate grade; --, active gland.

(c) *Transference from a high or a low temperature to an intermediate temperature.* In both cases the thyrotrophs showed some degree of activation, which was intensified by thiourea treatment. No difference could be discerned between the two groups.

(d) *Prolonged maintenance under experimental conditions.* The control and experimental fish showed no apparent difference. Those treated with thiourea showed progressively increased vacuolation and reduction of cell content until at the end of the experiment, i.e. after 12 weeks, the thyrotrophs could be discerned only as an empty sphere delimited by a faintly staining ring at the periphery.

Carassius. At low temperatures the thyrotrophs stained intensely and appeared full of inclusions. Neither increased temperature or immersion in thiourea caused any appreciable change in 28 days (Pl. 14 E).

Thyroid-pituitary relationship

Phoxinus. In specimens which showed intermediate thyrotroph activity there was no change in thyroid activity. In specimens showing active thyrotrophs, due either to exposure to a higher temperature or to immersion in thiourea, there was a slight increase in thyroid activity, the e/f rising by 10. In the case of prolonged immersion in thiourea the thyrotrophs reached the completely evacuated stage, whilst the thyroid activity underwent a rise and fall.

Carassius. The environmental changes to which the animals were subjected caused no change in either the thyrotrophic activity or the thyroid (Fortune, 1956) and both presented the normal resting picture.

DISCUSSION

Experimental evidence suggests that the presence of cellular contents in the thyrotrophs is indicative of stored TSH; consequently the emptying or breakdown of the contents can be interpreted as an increased secretion of TSH (Atz, 1953; Barrington & Matty, 1955). This interpretation is largely based on the fact that treatment with thiourea or thiouracil promotes these changes and the vacuolization of these cells becomes prominent, showing a similar reaction to mammalian thyrotrophs.

These changes are shown in *Phoxinus* with increased temperature and with thiourea treatment. The thiourea treatment is more rapidly effective at high temperatures. It can be stated that increased temperature causes increased secretion of TSH.

Following from this it would be expected that thyroid activity would also increase with increased temperature. Contradictory results have been reported. One possible explanation of these is that genuine specific differences exist. This seems to be the case in *Carassius auratus*, which has a thyroid resistant to external change, and explains some of the results where this species has been used as the only teleost representative. It may then be asked why such specific differences exist in related animals under similar environmental conditions. In the case of *C. auratus* the long period of artificial selection is probably adequate to explain many

abnormalities. Until reasons for a different endocrine response are forthcoming for other teleosts further explanation must be sought. One point that is not always sufficiently emphasized is that the range of temperature over which a response is sought must be related to the normal temperature range of the species, and no marked activation of the thyroid with increasing temperature can be expected until the temperature is approaching the upper lethal. Baggerman (1957) has put forward a similar suggestion in that there may be a critical level for each species below which no response to temperature takes place.

A further possibility is that the temperature to which the animal has been subjected prior to the experiment affects its response. This possibility is suggested by experiments on the oxygen consumption where fish kept at 20° C., with an induced high consumption, and fish kept below 12° C., with an induced low consumption, showed a reversal of this when brought to an intermediate temperature (Wells, 1935). Additional experiments, based on respiratory rate and susceptibility to lethal agents, showed that 'differences in physiological activity which had originally been induced by acclimatization to different temperatures were completely reversed when the fishes were brought to a common temperature' (Sumner & Dourodoff, 1938). Pretreatment for a fortnight, at extremes unlikely to be encountered under natural conditions, did not affect the thyroid activity as measured by histological changes. Variations in activity due to this cause may be all over in the first few days, which form a latent period for histological change; further, the method may not be sufficiently sensitive to detect these variations. It is, however, apparent that there is no long-term effect on thyroid activity due to pretreatment temperatures.

When the majority of thyrotrophs are activated there is a slight increase in the *e/f* ratio. It is known from previous experiments that this is the commencement of a gradual rise to a considerably higher *e/f* value. It seems that the thyrotrophs are more sensitive than the thyroid to external conditions and that changes in thyrotroph activity precede changes in thyroid activity. This may partly explain the fact that 'Ambinon' injections at a low temperature affected the thyrotrophs before any change became apparent in the thyroid. An additional factor here is that the TSH is accompanied by gonadotrophins which will also affect the endocrine balance.

One of the most interesting points to emerge was the change in the thyroid on prolonged treatment with thiourea. The epithelial height rose to a maximum and then declined with the reappearance of some colloid. This might be interpreted as adaptation of the gland to thiourea, or exhaustion of TSH causing decline in epithelial height. Neither possibility can be ruled out by the histology of the thyrotrophs. It does, however, indicate that the TH:TSH ratio can be set at different levels, for example high or low production of both hormones. It is known that under normal conditions the negative feed-back maintains the ratio constant. Under disturbed metabolic conditions, due either to temperature extremes or metamorphic changes, the reciprocal control between TSH and TH secretion rates becomes disturbed and, if successful adaptation ensues, becomes set at a new level which is expressed histologically. A changing histological picture would then

indicate active attempted adaptation, whereas a static picture would indicate successful adaptation to the prevailing conditions although actual hormone production might vary according to the setting of the ratio.

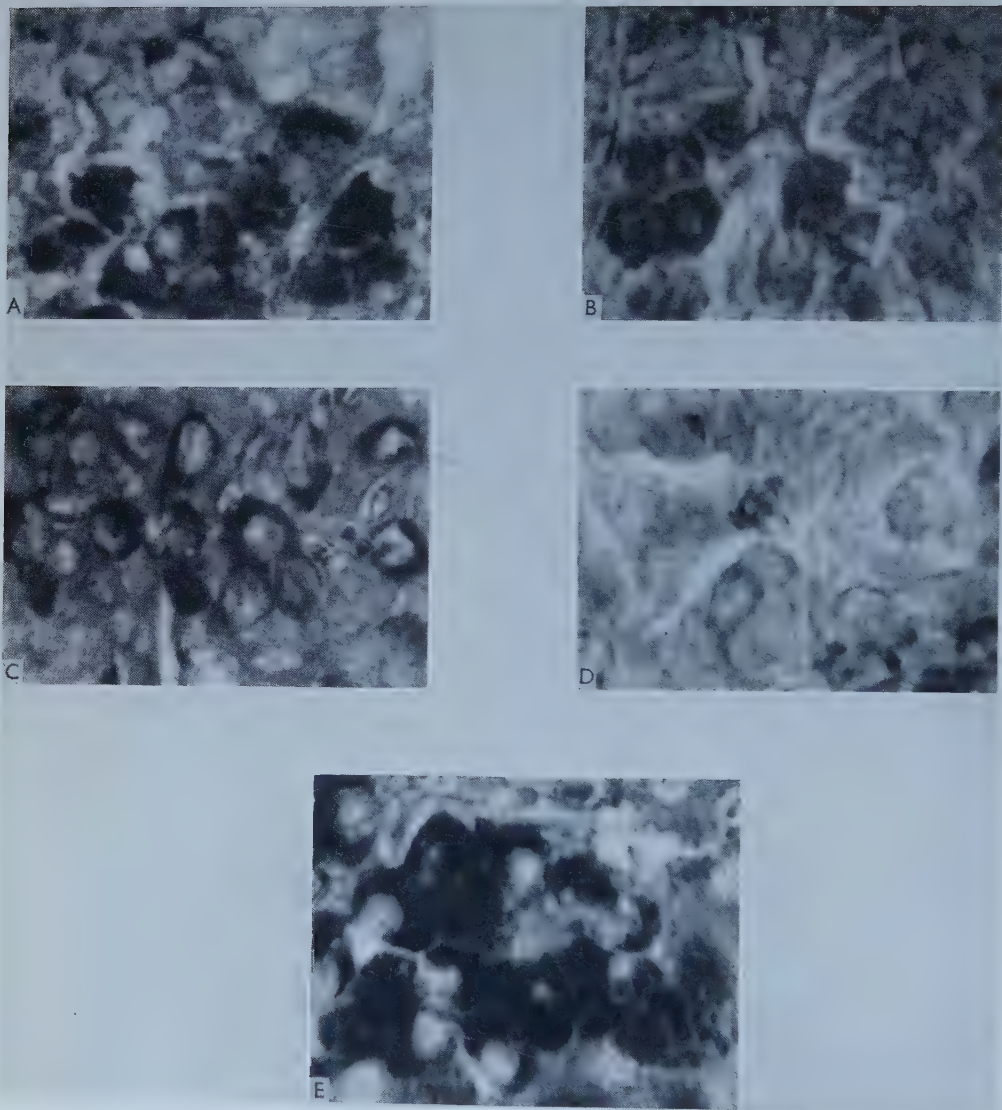
SUMMARY

1. It is confirmed that TSH has a more marked effect on the teleost thyroid at high temperatures.
2. The secretion of TSH is increased at high temperatures.
3. The temperature to which the animal is subjected prior to the experiment has no long-term effect on thyroid activity.
4. The thyroid-pituitary relationship is affected by temperature changes, and it is suggested that the TH:TSH ratio may be set at different levels by altering the environmental temperature.

I should like to thank Dr T. Kerr for much helpful discussion of the problem, and also Mr D. H. Jones who took the photographs. Dr J. W. Tindall of the Organon Laboratories, Ltd., kindly provided the 'Ambinon'.

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FORTUNE—THE EFFECT OF TEMPERATURE CHANGES ON THE THYROID-PITUITARY RELATIONSHIP IN TELEOSTS

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EXPLANATION OF PLATE

Phoxinus

- A. Inactive gland showing thyrotrophs with granular inclusions. Note a group of three at the bottom. Some gonadotrophs are present at the right. AF.
- B. Moderately active gland with thyrotrophs showing degranulation. Note reduction in cellular contents in the cells on the right. Taken after exposure to 25° C. for 14 days. AF.
- C. Moderately active gland. Same category as (b) but thyrotrophs less vacuolated, although showing some changes. Thiourea at 10° C. for 14 days. AF.
- D. Active gland showing empty and vacuolated thyrotrophs. Note a group of three at the bottom centre showing extreme emptying and appearing as faintly staining ring. Exposed to thiourea at 25° C. for 14 days. AF.

Carassius

- E. Inactive gland with heavily staining thyrotrophs. Exposed to thiourea at 25° C. for 28 days. AF.

THE EFFECTS OF TEMPERATURE AND OF EGG-LAYING ON THE LONGEVITY OF *DROSOPHILA SUBOBSCURA*

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(Received 16 May 1958)

1. INTRODUCTION

If an outbred population of adult *Drosophila* are kept from the time of emergence in a uniform and favourable environment there is a fairly protracted initial period during which very few individuals die, followed by a period during which the force of mortality rises rapidly until all individuals are dead. Similar life tables can be obtained for most animal species, provided that the environment is favourable and the population is neither genetically very diverse nor excessively inbred.

Such results show that progressive changes take place in individuals, starting at the time of emergence, and that these changes ultimately result in death or render individuals increasingly susceptible to various extrinsic causes of death.

As would be expected, in poikilotherms such changes proceed more rapidly at higher temperatures, as is shown by the decrease in the expectation of life with increasing temperature. It was the purpose of the present investigation to discover how far the processes responsible for death in *D. subobscura* are the same at different temperatures, differing only in the rate at which they proceed, and how far different processes are concerned at different temperatures. The results obtained strongly suggest that different processes are responsible for ageing at different temperatures; they also indicate a connexion between the rate of egg-laying and the rate of ageing, and this possibility has been confirmed by a study of ageing in virgin females and in females lacking ovaries.

2. METHODS

The flies used in these experiments (except for those described in §7) were first generation hybrids between the **K** and **NFS** inbred lines of *D. subobscura* (Maynard Smith, Clarke & Hollingsworth, 1955).

All flies were raised in half-pint milk bottles on a food medium of maize meal, agar and molasses, with two drops of living yeast suspension added, and at a temperature of 20° C. unless otherwise stated. On the day of emergence adults were removed from the culture bottles and kept subsequently in mated pairs in 3 in. by 1 in. diameter vials containing a similar food medium. They were transferred without etherization to fresh food vials at regular intervals, of 4 days if kept at 20° C., of 2 days at 25° C., of 1 day at 30.5° C. and of 12 hr. if kept at 33° C. Deaths were recorded only at the time of transfer (except at 33° C., when they were recorded at 3-hourly intervals) and for purposes of calculation were assumed to have taken place mid-way between successive transfers.

3. SURVIVAL TIMES AT DIFFERENT TEMPERATURES

The mean ages at death (measured from adult emergence) of adults kept continuously at 20°, 25°, 30·5° and 33° C. are given in Table 1.

Table 1. Mean survival times in days of adult flies at various temperatures

Temperature (°C.)	Males		Females	
	No. of flies	Survival time in days	No. of flies	Survival time in days
20	50	67·4 ± 2·46	50	55·9 ± 2·58
25 { raised at 15° C. raised at 25° C.	25	29·5 ± 1·07	25	40·5 ± 1·68
	25	24·6 ± 1·10	25	30·6 ± 1·65
30·5	50	7·58 ± 0·28	50	17·6 ± 0·65
33	10	0·79 ± 0·08	10	0·82 ± 0·05

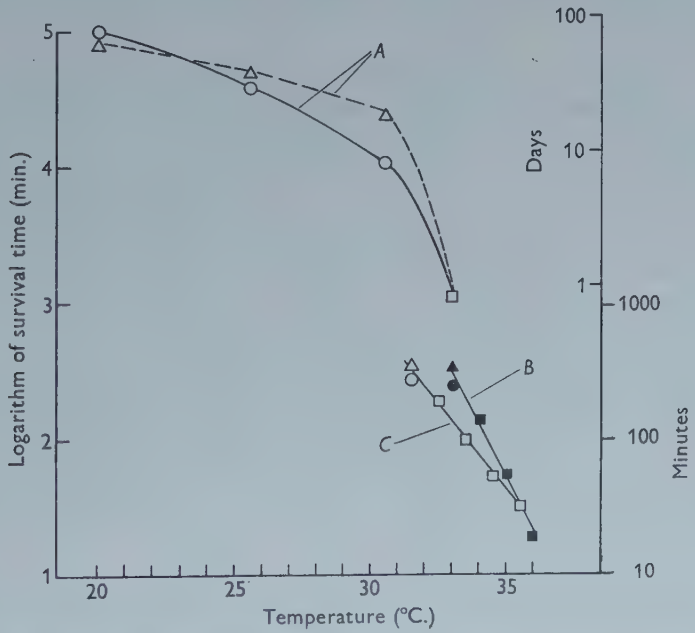


Fig. 1. Survival time of flies at different temperatures. A, In food vials; B, in saturated air; C, in dry air; Δ , \blacktriangle , females; \circ , \bullet , males; \square , \blacksquare , sexes combined.

In Fig. 1 these values are plotted on a logarithmic scale, together with the results of earlier experiments (Maynard Smith, 1957) on F_1 hybrids between the B and K inbred lines exposed to high temperatures without food or water in dry and in saturated air. It is known (Maynard Smith, 1956b) that at these high temperatures the survival times of B/K and of K/NFS hybrids do not differ significantly.

There is a rather sudden change in the slope of the curve in Fig. 1 at about 31° C. This suggested that the causes of death at high temperatures might be different

from those acting below 31°C . This is confirmed by two other differences between the factors influencing survival time at high and at low temperatures.

First, flies raised during pre-adult life at 25°C . survive at 33.5°C . in dry air for about twice as long as do flies raised at 15°C . There is a similar, although smaller, difference between the survival times of the two groups at 34.3°C . in saturated air. But precisely the opposite is true of survival at 25°C . As shown in Table 1, flies raised at 15°C . live for longer as adults than do flies raised at 25°C . Hence the process of acclimatization in larvae raised at 25°C . enables them to live for longer at temperatures above 33°C ., but not at 25°C .

Table 2. *Mean total survival times in hours at 33°C . in food vials*

	Males	Females
Kept continuously at 33°C .	18.85	19.55
Kept alternately for 8 hr. at 33°C . and for 16 hr. at 20°C .	28.45	26.05

A second and more important difference concerns the reversibility of changes occurring at temperatures above and below 31°C . It was found (Maynard Smith, 1957) that if flies are exposed to a high temperature (33.5°C . in dry air or 34.3°C . in saturated air) for 50 min. (i.e. for about half their expectation of life at that temperature) and are then kept for 3 hr. at 20°C . in a food vial, their survival times when they are again exposed to a high temperature are as great as those of flies not previously exposed. (In fact, they live for longer in their second exposure, but this only strengthens the argument.) It follows that whatever damage was done during the first exposure can effectively be repaired during the intervening period at 20°C ., and in this sense the changes which occur at high temperatures are reversible. The same is in part true of the changes which kill individuals kept in food vials at 33°C ., as is shown in Table 2. Although the changes which occur in 8 hr. in a food vial at 33°C . are not completely reversed, some recovery does take place during the intervening periods of 16 hr. at 20°C . It will now be shown that this is not true of the changes which occur at 30.5°C .

4. IRREVERSIBILITY OF CHANGES OCCURRING AT 30.5°C .

If the changes which occur at 30.5°C . and which ultimately are responsible for death at that temperature were in part reversible, or if the damage caused could be repaired, then it would be expected that the total survival time at 30.5°C . would be greater for flies exposed intermittently, with intervening periods at 20°C ., than for flies exposed continuously. Table 3 shows that there is little sign of such reversibility.

In the case of males there is evidence for a small degree of recovery, since the first 8-day interruption at 20°C . did slightly increase the further expectation of life at 30.5°C ., although the second 8-day interruption did not. In the case of females flies exposed intermittently had total survival times at 30.5°C . which were if anything slightly shorter than those of flies exposed continuously.

It is concluded that, at least in the case of females, the changes responsible for death at 30.5° C. cannot to any appreciable extent be reversed or repaired at 20° C.

Table 3. *Expectation of life at 30.5° C.*

	No. of flies	Further expectation of life at 30.5° C. days
Females		
(1) Exposed continuously to 30.5° C.	50	17.60 ± 0.65
(2) After 5 days at 30.5° C.		
(a) Exposed continuously	50	12.60 ± 0.65
(b) 8-day interruption at 20° C. after 5 days at 30.5° C.	25	11.02 ± 0.28
(3) After 13 days at 30.5° C.		
(a) Exposed continuously	44	5.82 ± 0.49
(b) 8-day interruption at 20° C. after 5 days at 30.5° C.	25	3.02 ± 0.28
(c) two 8-day interruptions at 20° C. after 5 and 13 days at 30.5° C.	22	5.23 ± 0.32
Males		
(1) Exposed continuously to 30.5° C.	50	7.58 ± 0.28
(2) After 5 days at 30.5° C.		
(a) Exposed continuously	49	2.64 ± 0.27
(b) 8-day interruption at 20° C. after 5 days at 30.5° C.	44	5.23 ± 0.38
(3) After 8 days at 30.5° C.		
(a) Exposed continuously	21	1.40 ± 0.35
(b) 8-day interruption at 20° C. after 5 days at 30.5° C.	36	2.97 ± 0.36
(c) Two 8-day interruptions at 20° C. after 4 and 8 days at 30.5° C.	25	2.42 ± 0.23

5. THE EFFECTS ON THE EXPECTATION OF LIFE AT 20° C. OF A PREVIOUS EXPOSURE OF ADULTS TO 30.5° C.

Since the changes responsible for death at 30.5° C. are not reversed at 20° C., it seemed possible that the same processes might ultimately be responsible for death at the two temperatures. If the ageing processes were in fact identical a simple relationship would hold for total life span, whereby a period of n days at 30.5° C. would reduce the expectation of life at 20° C. by $n \times 55.9/17.6$ days for females, and by $n \times 67.4/7.58$ days for males. Although slight differences between the ageing processes at the two temperatures might upset this simple relationship, it would still be expected that exposure to 30.5° C. would reduce the expectation of life at 20° C.

Experiments in which flies were kept for varying periods at the two temperatures do not confirm this simple hypothesis. Fig. 2 and Table 4 show the results of exposing young adult females to 30.5° C. for 5, 8 and 12 days (i.e. for 28, 45 and 68 % of their expectation of life at that temperature) and then keeping them at 20° C. until they died. So far from reducing their expectation of life this exposure in fact increased it, by as much as 50 % in the case of females exposed for 8 days. These results are fully confirmed by a similar experiment to be described in §7.

A similar experiment on males (Table 5 and Fig. 3) gave slightly different results. A group of males were exposed to 30.5°C . for 5 days, i.e. for 66% of their expectation of life at that temperature. Four males died almost immediately after their

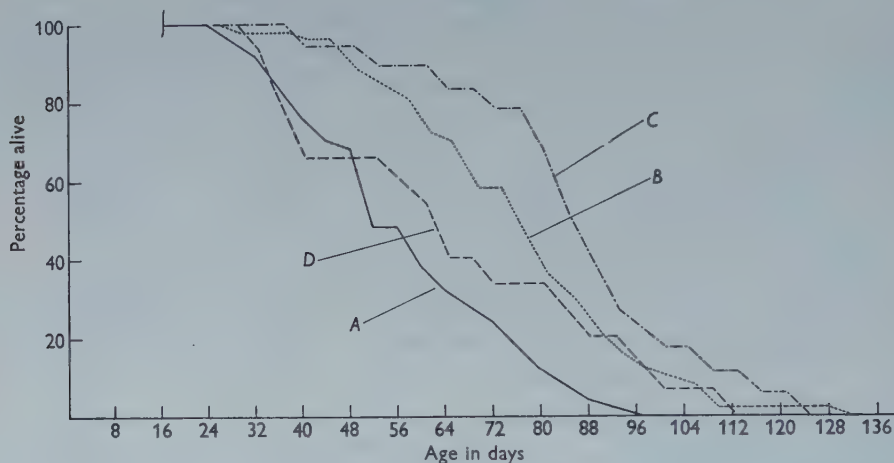


Fig. 2. Survival time at 20°C . of females previously exposed to 30.5°C . A, Unexposed; B, exposed for 5 days; C, exposed for 8 days; D, exposed for 12 days.

Table 4. *Expectation of life in days of females kept at 20°C .*

	No. of flies	Further expectation of life in days at age 17 days
Kept continuously at 20°C .	50	38.9 ± 2.6
Exposed to 30.5°C . for 5 days (6th to 10th day after emergence)	47	57.2 ± 3.0
Exposed to 30.5°C . for 8 days (6th to 13th day after emergence)	18	67.8 ± 4.9
Exposed to 30.5°C . for 12 days (6th to 17th day after emergence)	15	50.0 ± 6.6

Table 5. *Expectation of life in days of males kept at 20°C .*

	No. of flies	Further expectation of life in days at age 10 days	No. of flies	Further expectation of life in days at age 14 days
Kept continuously at 20°C .	50	57.4 ± 2.5	50	53.4 ± 2.5
Exposed to 30.5°C . for 5 days (6th to 10th day after emergence)	47	51.5 ± 3.6	43	53.4 ± 2.8

return to 20°C . If these are ignored, as in the last column of Table 5, the expectation of life of the remainder at 20°C . did not differ from that of the unexposed controls. Thus the simple additive assumption outlined above is again contradicted by the results, but in this case there is no prolongation of life due to exposure; these findings are again confirmed by an experiment to be described in §7.

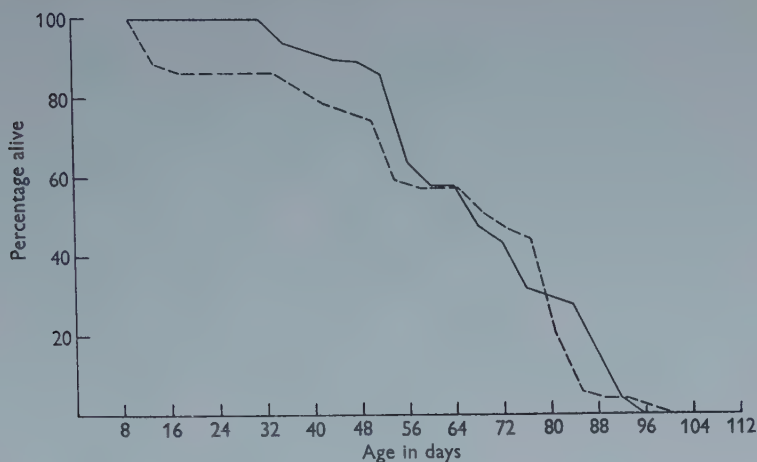


Fig. 3. Survival time of males at 20° C. Full line, unexposed; broken line, exposed for 5 days to 30.5° C.

6. OTHER EFFECTS OF EXPOSURE TO 30.5° C.

(a) Male mating behaviour and fertility

Twenty-five males were kept for 5 days at 30.5° C. and paired with virgin **B/K** females immediately after being returned to 20° C. Nineteen of these pairs were seen to mate normally within 1 hr. Eggs were collected from the nineteen mated females, with the results shown in Table 6. Exposure to 30.5° C. did not seriously impair the mating ability of these males, nor, with the exception of one mating, did it greatly reduce their fertility.

Table 6. Fertility of flies after exposure to 30.5° C. for 5 days

	No. of females	No. of eggs		
		Laid	Hatched	Unhatched
Females exposed and then mated	9	93	85	8
Females mated and then exposed	20	216	191	25
Females mated to exposed males	18	187	182	5
	1	16	0	16

(b) Female mating behaviour and fertility

Twenty virgin females were exposed for 5 days to 30.5° C., and paired with **B/K** males immediately after their return to 20° C. In no case did mating take place within 1 hr., and the females were seen to extrude their ovipositors towards approaching males, a signal normally characteristic of inseminated females (Maynard Smith, 1956a). These twenty pairs were kept in the dark (in which mating does not take place) and tested in the light for 1 hr. daily for the next 12 days. Seven pairs mated, apparently normally, during the next 4 days and two more mated on the

tenth day. The remaining eleven females did not mate and continued to extrude their ovipositors. These eleven females were dissected; as expected, they were found not to contain sperm. The state of development of their ovaries was variable but the ovaries were always smaller than those of unexposed females. This means that there was a regression of the ovaries during exposure to 30.5°C ., since the females had been kept for 5 days at 20°C . before exposure and at that age their ovaries would have been fully developed.

This experiment was repeated with 'ovariless' females from the *grandchildless* stock (Spurway, 1948). It was found that 'ovariless' females do not mate for 2 days after emergence, mate readily 4 days after emergence, extrude their ovipositors at an approaching male once they have been inseminated, and that after 5 days at 30.5°C ., even if virgin, they behave like fertilized females, extruding the ovipositor and refusing to mate. It seems therefore that exposure of normal females to 30.5°C . causes a partial regression of the ovary and produces a long-term change in behaviour, similar to that caused by the stimulus of insemination, but that the change of behaviour is not caused by changes in the ovary since an exactly similar change in behaviour occurs in 'ovariless' females.

Eggs were collected from the nine exposed virgin females which did mate, and also from twenty females which mated before being exposed for 5 days to 30.5°C . The results are shown in Table 6. A high proportion of the eggs laid by exposed females hatch and the second experiment shows that sperm stored in a female's receptacle is not seriously damaged by exposure to 30.5°C .

(c) *Female productivity*

Since it is known that about 90% of the eggs laid by exposed females hatch, a rough estimate of the rate of egg-laying can be obtained by keeping such females in food vials for a day and counting the number of adult offspring which emerge. The 'mean productivity' (i.e. the number of adult offspring which emerged from a day's lay of eggs, divided by the number of surviving females) was measured for a group of twenty-two females, mated to **K/NFS** males and then exposed to 30.5°C . for 5 days; measurements were made at intervals until the females were 63 days old. In Fig. 4, the productivity of these twenty-two females is compared with that of eleven unexposed females measured in a similar manner.

No offspring were obtained from the exposed females for the first few days after their return to 20°C ., and tests on other females showed that no eggs are laid during this period. But from the 12th to the 30th day after their return to 20°C . all females were producing at least a few offspring. However, the productivity of the exposed females was always lower than that of the controls, and this almost certainly reflects a lower rate of egg-laying.

7. THE RATE OF EGG-LAYING AND THE RATE OF AGEING

Since the expectation of life of females, but not of males, was increased by exposure to a high temperature, and since that exposure also caused a partial regression of the ovaries and a permanent reduction in the rate of egg-laying, it seemed possible

that the latter changes were the direct cause of the former. That is, unexposed females may die sooner because they lay eggs more rapidly.

Therefore two other ways of reducing the rate of egg-laying by females were investigated. The first is to keep the females unmated; virgin females do lay eggs, but at a greatly reduced rate (Maynard Smith, 1956*a*). The second is to use females without ovaries. Females homozygous for the mutant *grandchildless* lay eggs which develop into adults without gonads. The testes in this species have an orange sheath, and can be seen through the body wall. Thus a female can be recognized as *gs/gs* because her sons have no testes; it can then be assumed that her daughters have no ovaries, without dissecting the daughters. The stock which segregates for *gs* is necessarily highly inbred. Therefore virgin females from this stock were crossed to **K** males, and outbred 'ovariless' females obtained from the F_1 . As normal controls, offspring from similar crosses in which the female parent proved not to be *gs/gs* were used.

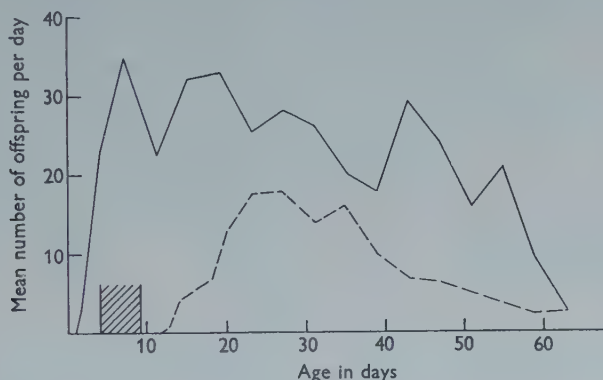


Fig. 4. Full line, mean productivity of eleven females kept continuously at 20° C.; broken line, mean productivity of 22 females exposed for 5 days to 30.5° C.

Life tables were obtained for the following groups of flies: (i) normal mated females; (ii) normal virgin females; (iii) 'ovariless' females; (iv) normal mated females exposed to 31° C. for 5 and for 6 days; (v) 'ovariless' females exposed to 31° C. for 3 days; (vi) normal mated males; and (vii) normal mated males exposed to 31° C. for 3 and for 4 days.

Two virgin females were kept in each vial; all other vials contained a male and a female.

The fact that flies were exposed to 31° C., and not to 30.5° C. as before, was due to an error; the periods of exposure were made correspondingly shorter. 'Ovariless' females were exposed for a shorter time than normal females because preliminary experiments suggested that their expectation of life at high temperatures was lower.

The results are given in Fig. 5 and Table 7. As before, a few males died immediately after their return to 20° C., but if these are ignored exposure for 3 days to 31° C. did not alter the expectation of life at 20° C. although exposure for 4 days did slightly reduce it.

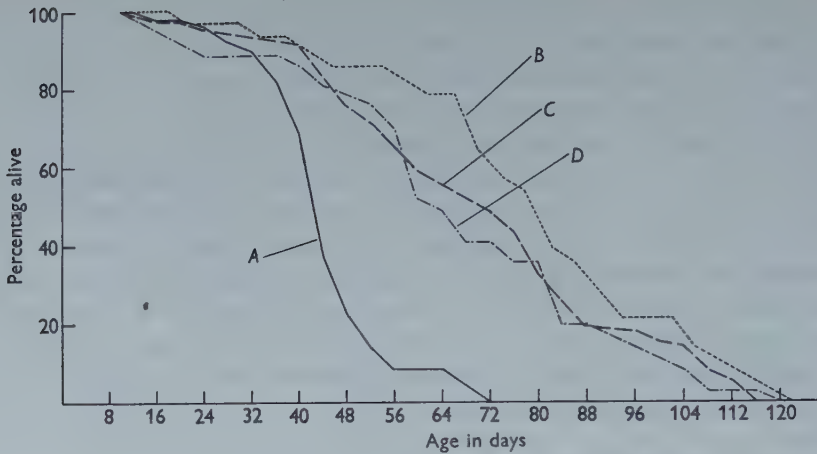


Fig. 5. Survival time of females at 20° C. *A*, Mated females; *B*, 'ovariless' females; *C*, virgin females; *D*, mated females exposed for 5 or 6 days to 31° C.

Table 7. *Expectation of life in days of flies kept at 20° C.*

	No. of flies	Further expectation of life in days at age 10 days	No. of flies	Further expectation of life in days at age 20 days
Males				
Kept continuously at 20° C.	79	41.1 ± 1.7	76	32.6 ± 1.6
Exposed to 31° C. for 3 days	39	36.3 ± 3.5	31	34.8 ± 1.8
Exposed to 31° C. for 4 days	26	23.8 ± 3.6	17	24.4 ± 3.2
Mated females				
Kept continuously at 20° C.	48	33.1 ± 1.6		
Exposed to 31° C. for 5 days	23	61.2 ± 5.7		
Exposed to 31° C. for 6 days	14	45.2 ± 5.7		
Virgin females				
Kept continuously at 20° C.	89	58.7 ± 2.7		
'Ovariless' females				
Kept continuously at 20° C.	28	67.6 ± 4.7		
Exposed to 31° C. for 3 days	22	64.2 ± 5.4		

The expectation of life of normal females was again significantly increased by exposure to 31° C. Both virgin and 'ovariless' females lived for longer than the controls and closely resembled females exposed to a high temperature. Finally, the expectation of life of 'ovariless' females was not increased by exposure to 31° C. In this respect 'ovariless' females resemble males and not normal females.

It is therefore reasonably certain that the hypothesis outlined above is correct; the expectation of life of females can be increased by reducing the rate of egg-laying, either by keeping them unmated, or by using 'ovariless' females, or by exposing normal females to a high temperature for a period sufficient to cause a partial regression of the ovaries. The failure to increase the life span of 'ovariless' females by exposure to a high temperature seems decisive in showing that the prolongation of the life of normal females by such exposure is the direct result of the effect of high temperature on their ovaries, and so on the rate of egg-laying.

8. DISCUSSION

There is other evidence that in insects the longevity of females is reduced by laying eggs. Bilewicz (1953) found that in *D. melanogaster* virgin females lived for approximately twice as long as mated females but laid about the same total number of eggs in a lifetime; unmated males also lived longer than mated ones but the difference was very small. Similarly, Griffiths & Tauber (1942) found that virgin females of *Periplaneta americana* laid eggs less rapidly and lived for about 60% longer than did mated females. Rockstein (1957) found that the longevity of female houseflies, but not of males, was increased by adding powdered milk to a diet of sugar and water. Since the females on sugar and water only laid at least some eggs, this last observation suggests that in an egg-laying female various substances may be utilized more rapidly than they can be assimilated or synthesized and that this may accelerate ageing.

The other main point of interest concerns the causes of ageing at different temperatures. It seems reasonable to regard the changes which ultimately kill individuals kept at 30.5° C. as processes of ageing, since they are not reversible at 20° C. and since they take an appreciable time to reach completion (17.6 days for females as compared to 55.9 days at 20° C.). Yet these changes are not the same as those responsible for ageing at 20° C., since if they were the same, exposure to 30.5° C. would reduce the further expectation of life at 20° C. This conclusion holds both for males and for females. It follows that different processes are responsible for ageing and ultimately for death at the two temperatures.

9. SUMMARY

1. The adult life span of *Drosophila subobscura* has been measured at temperatures varying from 20° to 33° C. A sharp increase in the slope of the curve of log survival time against temperature occurs at temperatures above 31° C.

2. Changes which occur in individuals at 33° C. or above are reversible, at least in part, at 20° C.; but changes occurring at 30.5° C. are irreversible in the sense that the total survival time at 30.5° C. is not increased by intervening periods at 20° C.

3. Exposure of young adult flies to 30.5° C. for a period of about half their expectation of life at that temperature significantly increases the further expectation of life of females at 20° C. but does not alter the expectation of life of males. Such exposure causes a partial regression of the ovaries of females, a permanent change in their behaviour and a reduction in their rate of egg-laying; exposure does not alter the behaviour or seriously reduce the fertility of males.

4. 'Ovariless' females and virgin females live for significantly longer than do normal mated females. The expectation of life of 'ovariless' females at 20° C. is not altered by exposure to 30.5° C. It is concluded that egg-laying accelerates the ageing of females at 20° C., and that the prolongation of life of females exposed to 30.5° C. is due to the reduction in the rate at which such females subsequently lay eggs.

This work was supported by a capital grant from the Nuffield Foundation, whose assistance is gratefully acknowledged.

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ACID PRODUCTION AFTER FERTILIZATION OF SEA-URCHIN EGGS

A RE-EXAMINATION OF THE LACTIC ACID HYPOTHESIS

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(Received 11 June 1958)

INTRODUCTION

Sea-urchin eggs produce acid, the fertilization acid, for a short time after fertilization. Runnström (1933) was the first to describe this phenomenon; he found an acid production equivalent to about 150 μ l. CO_2 /ml. eggs for the first 20 min. after fertilization in *Paracentrotus lividus*. According to Laser & Rothschild (1939), the R.Q. of sea-urchin eggs (*Psammechinus miliaris*) is about 0.65 immediately after fertilization and, using this value, they observed an acid production equivalent to 150 μ l. CO_2 /ml. eggs during the first 10 min. after fertilization. This figure is based on the curves in fig. 6 of Laser & Rothschild's paper, though reference to the rate curves in the same paper and Rothschild's experiments (1956, fig. 9) shows that even higher rates of acid production may occur during the first 5 min. after fertilization.

Runnström's results and those of Laser & Rothschild were obtained by manometric methods. The positive pressures observed after fertilization of sea-urchin eggs in the absence of KOH were assumed to be due to the evolution of CO_2 following the diffusion of acid out of the eggs and its reaction with bicarbonate in the surrounding sea water. For a number of reasons, including the reported pH, 6.6, of echinoderm egg cytoplasm (Needham & Needham, 1926; Chambers & Pollack, 1927), the evolution of excess CO_2 after fertilization is unlikely to be caused by the production of an acid within the egg and its reaction with bicarbonate within the egg. This question can, however, be settled by experiments, which are described in this paper; they show that the sea water surrounding fertilized sea-urchin eggs has a markedly reduced bicarbonate content.

Runnström (1933) said that the fertilization acid was not lactic acid. Örström & Lindberg (1940), using unpublished results of Runnström, reported values of 0.97 ± 0.07 mg. lactic acid/100 mg. unfertilized egg N and 0.99 ± 0.1 mg. lactic acid/100 mg. fertilized egg N (5–12 min. after fertilization), which quantitatively confirmed Runnström's observations. Yčas (1950) also failed to find any increase in lactic acid production after fertilization. Some years ago I came to the same conclusion and, like other workers presumably, failed to identify the fertilization acid in spite of intensive investigations. The possibility of it simply being H_3O^+ has been discussed elsewhere (Rothschild, 1956, pp. 65–6).

In the light of these observations, a recent paper by Aketa (1957), in which the fertilization acid is claimed to be lactic acid in the eggs of the Japanese sea-urchin, *Hemicentrotus pulcherrimus*, is of special interest. Aketa obtained a maximum of 120 μg . lactic acid*/mg. protein N in the first 2 min. after fertilization. Aketa stated that the period of maximum lactic acid production occurred 2–5 min. after insemination and that after this the production rapidly fell. He said that the most probable reason why previous workers had failed to identify the fertilization acid was because they had not made their lactic acid estimations soon enough after fertilization. Later, e.g. 10–20 min. after fertilization, the lactic acid is apparently oxidized. This argument is difficult to reconcile with the fact that acid production, as evidenced by the evolution of CO_2 from sea water, continued for 20 min. in Runnström's experiments (1933), for at least 5 min. in those of Laser & Rothschild (1939) and for 15 min. in the experiment illustrated by Rothschild in *Fertilization* (1956). These observations are made with the realization that at the low manometric shaking rates imposed by the fragility of sea-urchin eggs there may be some delay in the evolution of CO_2 from sea water.

Aketa's results raise further difficulties. The unfertilized eggs were inseminated at $t=0$; they were then centrifuged and the supernatant sea water was removed; at $t=2$ min., trichloroacetic acid (TCA) was added. As the supernatant sea water was discarded, Aketa must mainly have measured lactic acid *inside* the sea-urchin eggs and not in the surrounding medium. If this is so and, if it is true that the fertilization acid diffuses out of the eggs into the surrounding sea water, the lactate found by Aketa within the eggs was, in all probability, not the fertilization acid. The same conclusion can be reached in another way. Suppose that the fertilization acid is lactic acid and that it diffuses out of the egg at fertilization. Why should it disappear or be oxidized in sea water? Alternatively, if there is so much lactic acid within the eggs, why should the lactic acid in the sea water diffuse back into the eggs and be oxidized? These considerations make it unlikely that the fertilization acid is lactic acid, as Aketa believes; but his experiments emphasized the desirability of making a re-examination of this question, which is the subject of this paper.

MATERIAL AND METHODS

The eggs of *Echinus esculentus* were used, the jelly being removed by treatment with acidified sea water according to the method recommended by Hagström (1956). A concentrated egg suspension was prepared containing about 10^5 eggs/ml. sea water, i.e. 0.2 ml. eggs/ml., the radius of these eggs being *c.* 80 μ .† After testing the eggs for concentric fertilization membrane formation, 3 ml. of the suspension was

* Misprinted on p. 269 as 'mg. lactic acid'. In a letter dated 11 June 1958, Dr Aketa kindly gave me some information which indicates that 120 μg . lactic acid/mg. protein N is equivalent to about 1.4 mg. lactic acid/ 10^6 eggs, or 10 mg. lactic acid/100 mg. N, which is ten times Runnström's value, cited by Örström & Lindberg (1940).

† Dr H. Barnes kindly estimated the total N content of these eggs for me and obtained the value 67 mg. N/ 10^6 eggs. Ballentine (1940) found 5.8 mg. N/ 10^6 eggs of *Arbacia punctulata*, whose radius is 37 μ (Harvey, 1956). The ratio of these values, per ml. of eggs, is 1.01.

put into a conical Barcroft Warburg manometer vessel, vol. *c.* 20 ml., with 0.33 ml. of sperm suspension in the side-arm. There was no KOH in the centre cup. After the usual equilibration period at 16° C., the spermatozoa were mixed with the eggs, final sperm density 2×10^7 /ml., and readings were taken at 2, 5, 10 and 15 min. The experiment was discontinued unless significant acid production, as evidenced by a pronounced positive pressure, occurred. Assuming an R.Q. of 0.7 after fertilization, and that the ratio

$$\{\text{CO}_2 (\text{acid production}) + \text{CO}_2 (\text{respiration})\}/(\text{O}_2)$$

is 1.3 (Laser & Rothschild, 1939), it can be shown (see Appendix) that with the constants applicable to a manometer vessel of standard size containing 3.33 ml. fluid ($k_{\text{CO}_2} = 1.6$, $k_{\text{O}_2} = 1.4$) any positive pressure observed will be about one-third of the amount, in $\mu\text{l.}$, of the non-respiratory CO_2 evolved.

If the manometric pilot experiment was successful, in the sense that a pronounced positive pressure occurred after fertilization, 1 ml. of the parent egg suspension, which had been aerated meanwhile, was removed for an egg count. 10 ml. of egg suspension were then put into each of four 50 ml. beakers, called 1, 2, 3 and 4. Two of these were fertilized and treated with TCA 2 and 5 min. after insemination. The other two, the unfertilized egg controls, were also treated with TCA 2 and 5 min. after the beginning of the experiment. Table 1 shows how the experiment was done. The times after insemination at which TCA was added were varied according to the rate of acid production observed in the pilot experiments, again bearing in mind that the evolution of CO_2 from sea water is likely to lag behind acid production because of the low shaking rate, 90 c.p.m., which is necessary.

Table 1. *Procedure for examining the lactic acid production of fertilized and unfertilized eggs of Echinus esculentus. T° C., 16*

Egg suspension	1	2	3	4
Time (min.)				
0	+ Spermatozoa	+ Spermatozoa	—	—
2	+ TCA	—	+ TCA	—
5	—	+ TCA	—	+ TCA
6	—	—	+ Spermatozoa	+ Spermatozoa

Final concentrations, 2×10^7 sperm/ml. and 5.5 % TCA in each case.

In the experiment described above one ends up with: (i) fertilized eggs + spermatozoa fixed 2 min. after insemination; (ii) unfertilized eggs, fixed at the same time as (i), + spermatozoa; (iii) fertilized eggs + spermatozoa, fixed 5 min. after insemination; (iv) unfertilized eggs fixed at the same time as (iii), + spermatozoa. This material was homogenized in a tissue homogenizer (Umbreit, Burris & Stauffer, 1957, p. 171) after which it was centrifuged; the lactic acid content of the supernatant was estimated by the method of Barker & Summerson (1941). Standards containing the appropriate amounts of zinc lactate in sea water were freshly pre-

pared for each experiment and treated in the same way as the experimental material except that, for obvious reasons, homogenization was not necessary.

The mixing operations described above were done in the same way as those involving known sperm-egg interaction times (Rothschild & Swann, 1952; Rothschild, 1953). Both the fertilized and unfertilized eggs require continual agitation to ensure oxygenation.

RESULTS

Diffusion of acid out of fertilized eggs into medium. As mentioned in the Introduction, the conditions in which Aketa did his experiments were such that the lactic acid he measured after fertilization of the eggs had not diffused into the surrounding sea water. This raised the remote possibility that the fertilization acid does not diffuse out of the eggs and that the excess CO_2 evolved after fertilization comes from bicarbonate within the eggs and not from the sea water round them. To test this possibility, a suspension of jelly-free eggs was divided into two parts, each containing the same number of eggs in 10 ml. of suspension. At $t=0$, one suspension was added to 1 ml. of sperm suspension and the other to 1 ml. of sea water. The suspensions were agitated to the same extent as in a manometric experiment and, after 6 min., were transferred to centrifuge tubes and centrifuged for 1 min. in a hand centrifuge. 3 ml. of supernatant was removed from each tube and placed in the main compartments of manometer flasks whose side-arms contained 0.4 ml. 0.1N-HCl. After temperature equilibration, the acid was tipped in each case into the sea water and the manometers were violently shaken for 25 min. The results of two experiments are shown in Table 2. They show without doubt that after fertilization something, which must be an acid, diffuses out of these sea-urchin eggs and reacts with the bicarbonate in the sea water with evolution of CO_2 . If sea water has a salinity of 32.52‰, the molality of bicarbonate in it is 2.285×10^{-3} (Barnes, 1954), so that the theoretical CO_2 yield from 3 ml. is 152 μl . Violent aeration for many hours is needed to remove all the bicarbonate- CO_2 from acidified sea water, which is the main explanation of the difference between 152 μl . and the values in the second column of Table 2.

Table 2. *Bicarbonate content, expressed in μl . CO_2 , of 3 ml. of sea water round unfertilized (U) and fertilized (F) eggs of Echinus esculentus, seven minutes after insemination. $T^\circ\text{C}$, 16*

Exp.	U	F
1	129	84
2	138	96

Lactic acid production. The results of all experiments are given in Table 3, from which the following conclusions may be drawn:

(1) The difference between the amount of lactic acid produced by fertilized and unfertilized eggs is negligible.

(2) The amount of lactic acid produced by fertilized eggs during the first few minutes after fertilization is inadequate to account for the total acid produced during this time. Very roughly, lactic acid production would have to be increased by a factor of 5 to justify the contention that it is the fertilization acid. The discrepancy is all the more marked because the eggs of this sea-urchin produce less fertilization acid than do those of *Psammechinus miliaris*.

Table 3. Lactic acid and other acid production by fertilized (F) and unfertilized (U) eggs of *Echinus esculentus*. $T^{\circ}\text{C.}$, 16

(1) Exp. no.	(2) Time after fertilization (min.)	(3) $\mu\text{g. lactic acid/ml. eggs}$		(4) Column (3) F, as $\mu\text{l. CO}_2/3\text{ ml. egg}$ suspension.	(5) $\mu\text{l. CO}_2$ (acid) evolved/3 ml. egg suspension*
		F	U		
1	2	29	34	5	—
2	2	14	15	3	15 (5 min.)
3	5	16	14	2	23
4	5	19	19	3	16
	10	23	22	4	15
5	5	10	9	2	32
	10	8	6	2	17
6	2	16	16	5	1
	5	16	16	5	16
7	2	9	9	2	3
	5	15	15	3	25
8	5	19	12	3	15
	10	18	14	3	27

* See p. 845 and Appendix for the derivation of the figures in this column.

It is difficult to conceive of any reason why the experimental procedure described in this paper could be responsible for the difference between Aketa's results and those described above; one experiment was, however, done in exactly the same way as were Aketa's, described in the Introduction to this paper. In this experiment the lactic acid found in unfertilized eggs was 12 $\mu\text{g./ml.}$ of eggs and in fertilized eggs 9 $\mu\text{g./ml.}$ of eggs. The average values for unfertilized and fertilized eggs (Table 3) were 15 and 16 $\mu\text{g./ml.}$ of eggs. These results provide some support for the suggestion, made on p. 844, that such lactic acid as is formed in sea-urchin eggs does not diffuse out of them into the surrounding sea water. (The so-called retention of lactic acid is discussed by Umbreit *et al.* (1957) in *Manometric Techniques*.)

DISCUSSION

The experiments described in this paper show that there is a marked difference between the eggs of *Echinus esculentus* and *Hemicentrotus pulcherrimus* in regard to (a) the quantity of lactic acid produced by fertilized eggs, (b) the relative quantities of lactic acid produced by fertilized and unfertilized eggs (in *Echinus esculentus* the quantities are the same) and (c) the discrepancy between the observed production

of lactic acid and of fertilization acid. In the light of these findings, together with those of Runnström (1933), Örström & Lindberg (1940) and of Yčas (1950), the conclusion is inescapable that the fertilization acid is not lactic acid, even if this substance is produced in unusually large quantities by the fertilized eggs of *Hemicentrotus pulcherrimus*.

SUMMARY

1. When sea-urchin eggs are fertilized an acid, the fertilization acid, diffuses out of them into the surrounding sea water. A claim has recently been made that the fertilization acid may be lactic acid, which is oxidized shortly after its production.
2. There is no significant difference between the amounts of lactic acid produced by unfertilized and just-fertilized eggs of *Echinus esculentus*.
3. The amount of lactic acid produced by fertilized eggs of this species is too small to explain the evolution of CO_2 observed when the eggs are fertilized in manometer vessels.
4. The sea water round just-fertilized eggs was found to contain markedly less bicarbonate than the sea water round unfertilized eggs, confirming that an acid diffuses out of the eggs after fertilization.
5. There is some evidence that the small quantities of lactic acid produced by unfertilized and just-fertilized eggs of this species do not diffuse into the surrounding sea water.
6. It is concluded that the fertilization acid is not lactic acid.

I am indebted to Prof. J. Runnström and Dr H. Laser for commenting on the typescript of this paper. This work is supported by the Medical Research Council.

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APPENDIX

Relationship between acid production, as $\mu\text{l. CO}_2$, and manometer reading

The following assumptions or definitions are made:

- (1) $k_{O_2} = 1.40$ and $k_{CO_2} = 1.63$,
- (2) $CO_2(R)$ = respiratory CO_2 ,
- (3) $R.Q. = CO_2(R)/O_2 = 0.7$ (Laser & Rothschild, 1939),
- (4) $CO_2(A) = CO_2$ due to acid production = $+a \mu\text{l.}$,
- (5) $\frac{CO_2(R) + CO_2(A)}{O_2} = \frac{130}{70} = 1.86$ (Laser & Rothschild, 1939).

Hence
$$\frac{CO_2(R) + a}{O_2} = 1.86,$$

and
$$\frac{CO_2(R) + a}{10CO_2(R)/7} = 1.86,$$

and
$$7CO_2(R) + 7a = 18.6CO_2(R),$$

and
$$CO_2(R) = 0.605a.$$

The manometer reading, H , will be

$$H = +\frac{a}{1.63} + \frac{0.605a}{1.63} - \left(\frac{10}{7} \times 0.605a \times \frac{1}{1.40} \right) \\ = a/2.7.$$

The acid production, in $\mu\text{l. CO}_2$ is therefore 2.7 times the observed manometer reading.

ADDENDUM

Aketa (1957, pp. 273-5), says there is a marked increase in lactic acid content following the fertilization of Japanese sea-urchins and that 'This observation conflicts with those of other workers, who have been unable to detect any such change'; and that the difference may be ascribed to the fact that 'other investigators performed their estimations at longer intervals after insemination than the author did'. Referring to the fertilization acid, Aketa says 'it is still premature to deny the possibility of a relation between the manometric production of unknown acid and the lactic acid production shown in this paper'. On the basis of these remarks, I concluded that Aketa believed the fertilization acid was lactic acid. But in correspondence since the acceptance of this paper, Aketa told me he did not believe the fertilization acid was lactic acid, but that it would be unwise to deny this possibility.

THE ELECTRICAL AND MECHANICAL RESPONSES OF THE PROTHORACIC FLEXOR TIBIALIS MUSCLE OF THE STICK INSECT, *CARAUSIUS MOROSUS* BR.

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(Received 7 May 1958)

INTRODUCTION

Insect muscles are innervated by relatively few motor axons. Some or all of these axons may evoke different electrical and mechanical responses in the muscle fibres they innervate (see Hoyle, 1957*a*). Only one full analysis of the neuromuscular mechanisms of an insect muscle has been made, on the jumping muscle of the locust (Hoyle, 1955*a, b*). Hoyle found that this muscle was innervated by three different kinds of axons which could elicit four distinct types of electrical response in the muscle fibres. He designated these 'fast' and 'slow', following crustacean terminology. Other studies of insect neuromuscular mechanisms have either been restricted to descriptions of 'fast' responses (Hagiwara, 1953; Hagiwara & Watanabe, 1954); or were made with techniques incapable of revealing the finer details (Pringle, 1939; Roeder & Weiant, 1950). Hoyle (1957*a*) has criticized in certain respects Wilson's (1954) description of the neuromuscular mechanisms of the cockroach.

The purpose of this account is to present information about the electrical and mechanical responses of a normal walking muscle, the prothoracic flexor tibialis of the stick insect, *Carausius morosus* Br., obtained by modern methods.

ANATOMY AND HISTOLOGY

The flexor tibialis muscle is similar in organization in all three legs. Like the meta-thoracic extensor tibialis muscle of the locust (Hoyle, 1955*a*) it is composed of bundles of fibres, or 'muscle units', arranged pinnately with their origin on a central apodeme and their insertion on the lateral cuticle (Fig. 1). The muscle is much less bulky, and therefore has fewer muscle units and fewer fibres per muscle unit, than in the locust. There are about seventeen pairs of muscle units, each unit containing ten to twelve fibres, in the prothoracic muscle.

The muscle is innervated solely by the crural nerve. As this nerve passes into the femur, it divides to send branches to the extensor tibialis and retractor unguis muscles, but the body of the nerve continues through the femur adjacent to the flexor tibialis muscle. The body of the nerve gives off branches at intervals on either

* Present Address.

side, each branch innervating a pair of adjacent muscle units on the same side of the femur. The main body of the nerve proceeds into the tibia, and is presumed to carry sensory fibres from tibial and tarsal receptors.

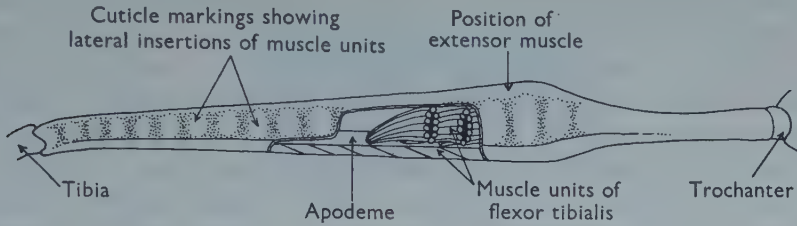


Fig. 1. Slightly ventro-lateral view of the prothoracic femur of *Carausius*. Cuticle partly removed to show some of the muscle units of the flexor tibialis, and two muscle units removed to show the position of the apodeme.

Each motor nerve branch which innervates a single muscle unit passes to the centre of the unit and there divides to send finer branches between and along the individual muscle fibres. These ultimately terminate in a number of motor nerve endings distributed at intervals of about 60μ along the muscle fibre they innervate. The main branch of the crural nerve contains a large number of axons of many different sizes, while the side branches to muscle units run obliquely and their detailed contents are therefore not very clear in transverse section. But in some cases two axons have been observed in the fine branches to individual nerve endings, and these presumably correspond to the two types of physiological response described in this paper. The motor endings are of the 'Doyère-cone' type, and in general appearance resemble the motor nerve endings in the locust (Hoyle, 1955*a*).

MECHANICAL PERFORMANCE OF THE MUSCLE

Hoyle (1955*b*) measured the tension developed under conditions of natural stimulation by the jumping muscle of the locust and concluded that it was about 1000 g./cm.^2 in terms of unit area of cross-section of individual fibres. This figure is similar to that for frog muscle, although the pinnate arrangement confers a much higher tension:weight ratio for the whole muscle, than in frog muscle and other vertebrate muscles where the muscle fibres are parallel.

The mechanical performance of the prothoracic flexor tibialis muscle of *Carausius* has been similarly examined. When lying ventral surface uppermost with its tarsi free in the air, as in the preparation used in this work, *Carausius* is very passive, and difficult to excite by natural stimulation. Stimuli were therefore applied to the crural nerve in the thorax at a frequency of about 20/sec. when testing mechanical performance. This frequency of stimulation produced a rapid tetanic contraction in the flexor. Weights were applied to the tip of the tibia by means of cotton thread, and the maximum weight which could be lifted during such a contraction was determined.

In a typical case the flexor of an adult insect was able to lift up to 2 g. At the latter weight flexion could be maintained only for about 1 sec., after which a period

of rest was necessary before the muscle could again be stimulated to lift the weight. This weight was therefore taken as the maximum load which the muscle was capable of lifting. The muscle weighed 9 mg., and the lengths of the tibia and femur were 1.8 and 1.85 cm respectively. Longitudinal sections showed the attachment of the apodeme to the head of the tibia to be about 0.3 mm. from the fulcrum. The lever factor was therefore approximately 60:1 and the peak tension developed by the muscle in lifting 2 g. was about 13,000 g./g. muscle weight, compared with 20,000 g./g. in the locust (Hoyle, 1955*b*). In terms of mean cross-section area of individual fibres the figure is about 800 g./cm.², compared with 1000 g./cm.² in frog and locust (Hoyle, 1955*b*). Variation between individuals might lessen the difference between figures for the two animals, but would be unlikely to abolish it.

The tetanus:twitch ratio was measured by a simple lever system. It was found to be over 25:1.

EXPERIMENTAL PROCEDURE

The electrical responses of the muscle fibres were studied by means of glass capillary intracellular microelectrodes. The preparation and procedure have been described previously (Wood, 1957). Mechanical responses were recorded with a simple electromechanical transducer (see Hoyle, 1955*b*). The output from the transducer was fed to the Y-plates of the oscillograph.

The two types of axons in the crural nerve were stimulated independently of one another by means of the anodal blocking technique of Kuffler & Vaughan Williams (1953). Impulses passing down the crural nerve were monitored from time to time, using tapered silver wire electrodes and an a.c. mains amplifier, also connected to the Y-plates of the oscillograph.

RESULTS

A single stimulus applied to the 'fast' (F) axon at above threshold level produced a quick twitch of the flexor tibialis muscle. This was accompanied by flexion of the tibia which, although brisk, was very slight in extent (Fig. 2A) and in most cases below the recording level of the mechanical transducer. Where recording was achieved increased tibial flexion was observed when the stimulus intensity was raised very gradually from threshold level, suggesting a recruitment of fibres of different thresholds. The increase in stimulus intensity and the increase in the mechanical response were so small that it is impossible to state with certainty the number of similar fibres present; but there appear to be at least four.

In the locust del Castillo, Hoyle & Machne (1953) found that when single shocks of high intensity were applied to the F axon they were followed by a tetanic contraction of the extensor tibialis muscle. This effect has not been observed in *Carausius*, in which a single shock of any intensity applied to the F axon results in a single twitch of the muscle.

The F electrical response has been described previously (Wood, 1957) and is shown in Fig. 3. In the insect's own haemolymph, containing 18 m. equiv.

potassium ions/litre, Wood (1957) found that the resting potential of six fibres averaged $41 \text{ mV.} \pm 1.5 \text{ (S.E.)}$ and the action potential of the same fibres averaged $39 \text{ mV.} \pm 2.2$. The action potential shows greater variation in magnitude from fibre

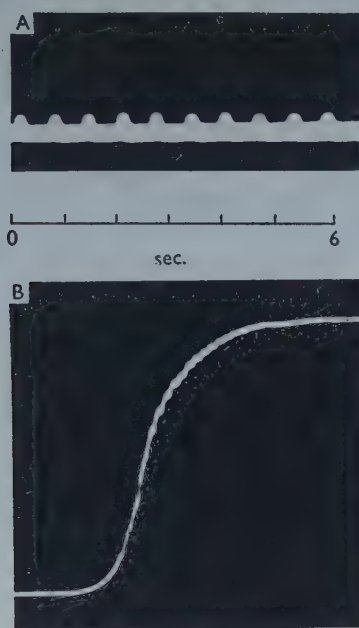


Fig. 2. Mechanical records of F responses. A, single twitches; B, tetanus resulting from repetitive stimulation at 20 stimuli per second. The time-scale refers to A, which is magnified by about $2 \times$ as compared with B.

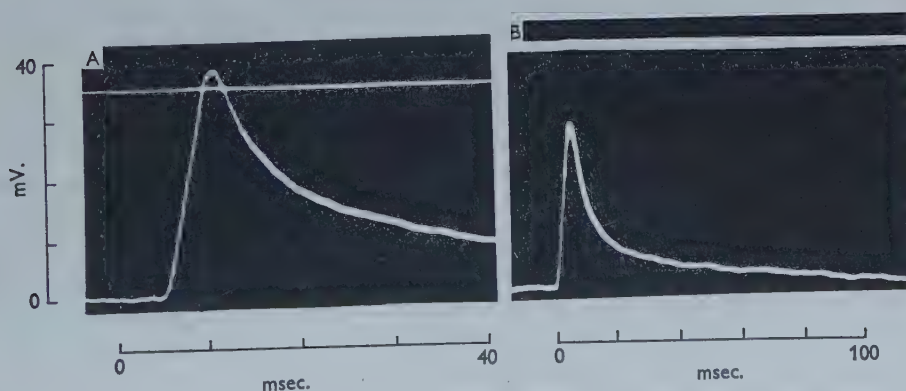


Fig. 3. Two examples, on different time bases, of F responses from two separate fibres of the same muscle.

to fibre than the resting potential. The rising phase occupies about 6 msec. and the rate of rise is about 5–7 V./sec. It is followed by a long decay phase of about 100–150 msec. The rising phase is composed of two components, the end-plate or

junctional potential and the spike or active membrane response. In many cases small overshoots of the active membrane response above the zero potential line are recorded. These never exceed about 8 mV. in size, and the majority are only a few mV. above the zero potential line.

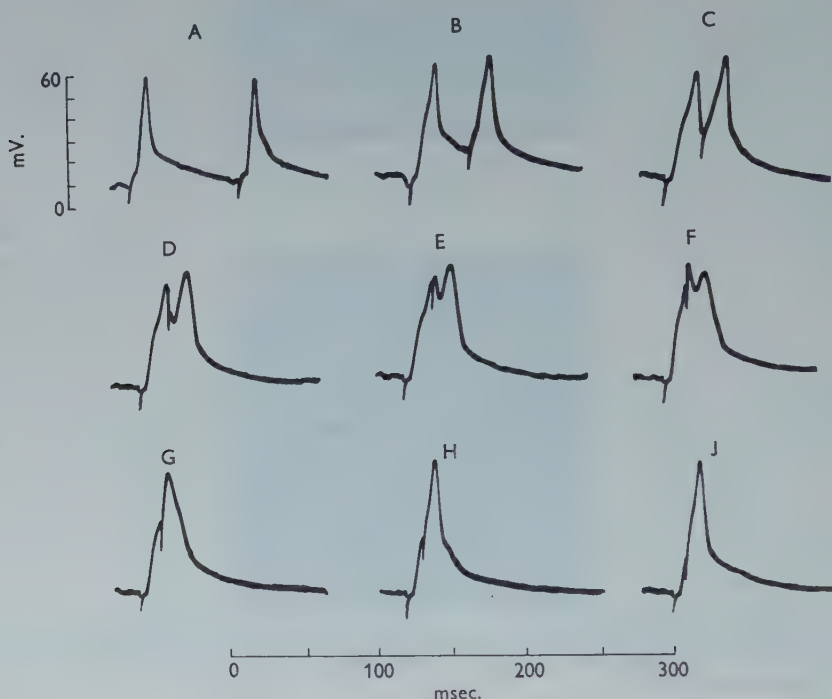


Fig. 4. The effect of paired stimuli on the F response. From A to J, the interval between the two stimuli was progressively reduced. Stimulus artifacts appear as small downward strokes.

Paired stimulation of the F axon

Stimuli were applied to the F axon in pairs, the interval between the two members of each pair being progressively reduced in successive experiments. The results are shown in Fig. 4. In Fig. 4A the stimuli were sufficiently apart in time for each to evoke a separate action potential. In Fig. 4B-D the second stimulus was applied at various times during the decay phase of the first response, and the second stimulus continued to elicit a second response, although the second action potential decreased in size as it approached the peak of the first action potential. In Fig. 4E-F the second stimulus was applied during the upper part of the rising phase of the first response, and in G-J during the lower part of its rising phase. In Fig. 4E-F the second response still falls in the decay phase of the first and continues to diminish in size as it approaches the peak of the latter. There may be a very small remnant in Fig. 4G but in H and J the second response has disappeared as a separate entity. However, in H and J the second stimulus was applied at or below the small inflexion which is presumed to mark the junction between the junctional potential and the

active membrane response; and although there is no separate second response the single action potential observed is larger in size than those resulting from single shocks. The single shock responses in Fig. 4A-F have a magnitude of about 42 mV., whereas the response in G measures about 53 mV., and in H and J about 60 mV. Judging by the position of the inflexion in the rising phase, the active membrane response remained constant in size, or nearly so, at about 20 mV. throughout the experiment. In H and J, and presumably in G, it is the junctional potential which increases in size.

Repetitive stimulation of the F axon

When the F axon is stimulated repetitively at frequencies above approximately 5/sec. a tetanic contraction is observed. At 5/sec. the tetanus is only partial, and individual twitches are clearly marked. Progressive fusion of these occurs up to a frequency of about 25 stimuli/sec.: a recording at 20 stimuli/sec. is shown in Fig. 2B. Above 25 stimuli/sec. the contraction is smooth. At this frequency the electrical responses are still discrete, and fusion of them does not begin until frequencies of about 50/sec. are approached. There is no evidence of facilitation while the electrical responses remain separate. Summation at high frequencies might be expected, in view of the experiments with paired stimuli; but this is offset by the tendency to fatigue at frequencies of about 20/sec. and above. This is shown by a progressive decrease in the ability to lift weights, and is often accompanied by a progressive diminution in size of the action potential (see Fig. 8).

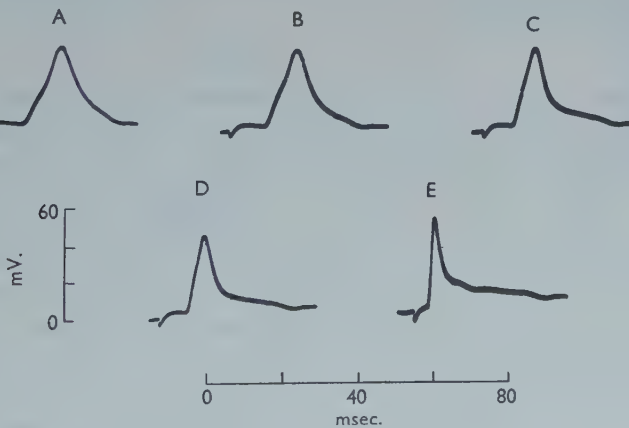


Fig. 5. Records of the F response at A, 5°; B, 10°; C, 15°; D, 20°; and E, 30° C.

Effect of temperature on the F response

Action potentials were recorded from muscle fibres bathed in salines at temperatures between 5° and 30° C. The electrode was kept in the same fibre throughout the experiment. As the temperature was lowered, the time course of the action potential lengthened, and the response became somewhat smaller in amplitude (Fig. 5). At the same time the latent period between the stimulus artifact and the onset of the action potential increased.

As the temperature was lowered progressively an inflexion appeared in the rising phase of the action potential (Fig. 5). This inflexion appears to mark the junction between the junctional potential and the active membrane response (Wood, 1957). The rate of rise of the two components changes at a comparable rate in the different temperatures. The inflexion therefore probably results from an increase in the time taken for the initiation of the active membrane response when the junctional potential reaches a critical threshold level, due to the decreasing rate of rise of the junctional potential as the temperature is lowered.

Effect of drugs on the F response

In an attempt to identify a possible neuromuscular transmitter, substances known to affect the excitability of nerves and muscles of other groups of animals were added to the bathing medium in concentrations effective in these animals, and the F responses were recorded. The test salines were allowed to bathe the muscle for at least 30 min. and in some cases for several hours. Substances tested in this way were atropine, sodium azide, 5-hydroxytryptamine, reserpine, chlorpromazine (given by Messrs May and Baker) strophanthin, histamine, acetylcholine, physostigmine, and noradrenalin. In the experiments involving the three latter drugs either 'hyalase' (a commercial preparation of hyaluronidase given by Messrs Bengers) or trypsin was added to the salines at concentrations of 1000 units/ml. and 0.1 % by weight respectively.

No significant alteration in the size or time course of the action potential occurred in any of these experiments.

The 'slow' response

Spontaneous activity is rare in this preparation but occasionally spontaneous movements of the tibiae, accompanied by electrical activity and contraction in the flexor muscle, have been observed. Some of these movements were brisk and appear from the records to have been due to F responses; but others were slower and smoother in character. Such slow, smooth movements were found to be associated with a second type of mechanical and electrical response, termed here the 'slow' (S) response. Whether spontaneous or evoked by single shock stimulation of the S axon (Fig. 6) the S electrical responses vary between 5 and 20 mV. in magnitude. In appearance they are very similar to junctional potentials and have an exponential decay phase. Their rate of rise is 1-1.5 V./sec., and although they may rise above the threshold level of the junctional response at which an active membrane response is normally initiated in the case of the F response, no inflexion has been observed in the rising phase of the largest S responses. This does not, of course, prove that no active component is present.

Repetitive stimulation of the S axon

If a single shock is applied to the S axon the muscle fibres can be seen to give a very faint twitch, but no movement of the tibia is detected. Several successive stimuli are necessary before a contraction occurs which will move the tibia.

When the S axon is stimulated at a sufficiently high frequency the S electrical

responses show a marked tendency toward facilitation (Fig. 7A). At frequencies of about 10–15 sec. these responses begin to fuse together (Fig. 7B). As the frequency of stimulation is raised, the extent and speed of contraction of the muscle increases. This may be coupled with an increase in depolarization of the membrane.

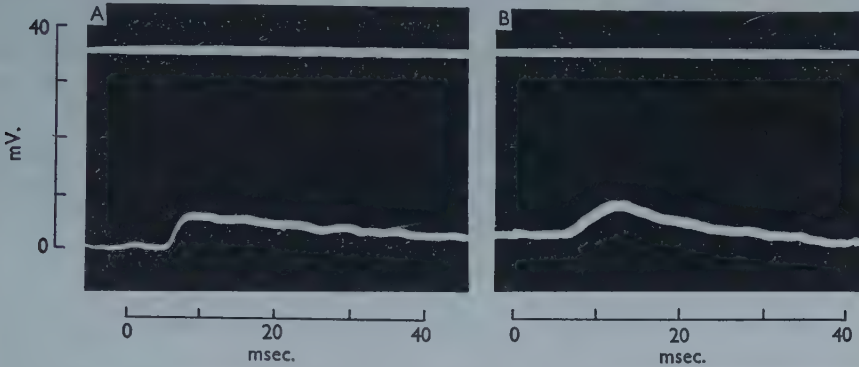


Fig. 6. Single S responses recorded: A, during spontaneous activity; B, during stimulation of the S axon.

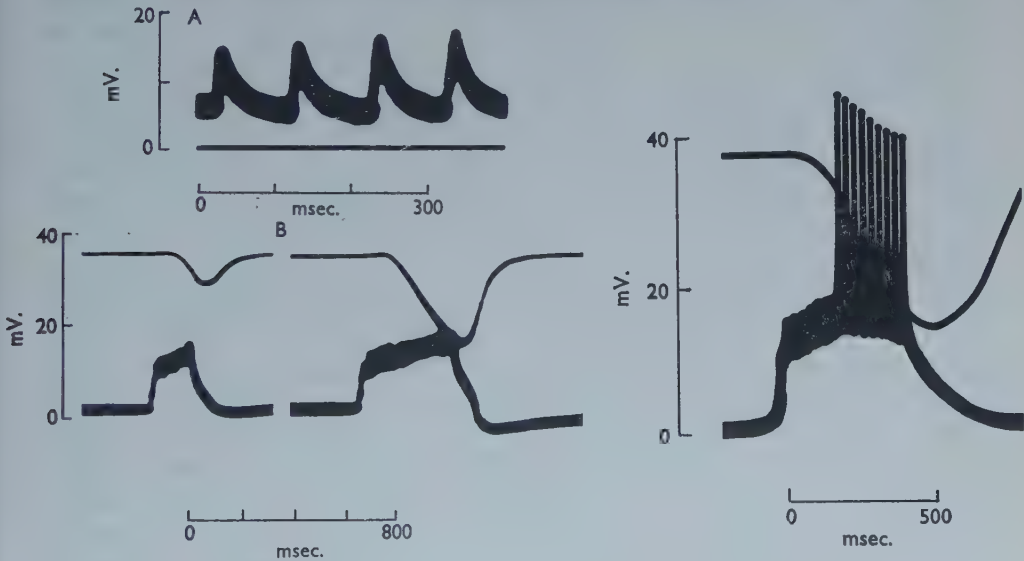


Fig. 7

Fig. 7. Repetitive stimulation of the S axon. A, facilitation at about 10 stimuli/sec.; B, summation of responses at 50 stimuli/sec. Upper trace, tension; lower trace, electrical responses (B only).

Fig. 8. Stimulation of the S axon, followed by simultaneous stimulation of the F axon, both at 40 stimuli/sec. Note the progressive decline of the F response at this frequency. Upper trace, tension; lower trace, electrical responses.

Occurrence of F and S responses

It is evident, both from observations made during spontaneous activity and from electrical stimulation of the crural nerve, that F and S responses can occur together

in the same muscle fibre. When this happens, F responses are simply superimposed upon the S depolarization (Fig. 8).

Over sixty experimental animals have been examined and over 4000 separate muscle fibres have been impaled at random during the course of this work, about 1000 in the metathoracic leg, and over 3000 in the prothoracic leg. Many of these fibres were superficial fibres in which the F responses only were studied, in connexion with previous work (Wood, 1957). F and S responses have been looked for at all positions and depths of the flexor muscles of different animals in only about 1000 fibres. This is an insufficient sample for definite conclusions to be drawn. Nevertheless, F responses have been found in every fibre impaled, and S responses in every fibre in which they have been looked for. It may therefore be tentatively concluded that all fibres of the flexor tibialis muscle are innervated by both F and S axons.

DISCUSSION

The resting potential of the muscle fibres of *Carausius* at 41 mV. is at the lower level of the normal range for insect muscle fibres. In insects, the highest value so far reported has been 60 mV. in *Locusta* and *Schistocera* (Hoyle, 1957*a*) and in *Gampsocleis* and *Platypleura* (Hagiwara & Watanabe, 1954); and the lowest value the figure of 42 mV. recorded by the latter authors in *Mecopoda* and *Graptosaltria*.

In the muscles of Acrididae the size of the action potential appears to be related to that of the resting potential, and there is less likelihood of an overshoot of zero potential in muscle fibres with low resting potentials (Hoyle, 1957*a*). Nevertheless such overshoots, although small in size, often occur in *Carausius*; and Wood (1957) found that they might persist in this insect even in potassium concentrations sufficient to reduce the resting potential to 20 mV. Hoyle (1957*a*) has shown that there is evidence that the extent of contraction of an insect muscle is related to the degree of depolarization of the muscle fibre membrane. Herbivorous insects typically have a high blood potassium concentration related to their diet (Boné, 1955; Duchâteau, Florkin & Leclercq, 1953) and Hoyle (1954) inferred that such insects might be condemned to sluggish movement owing to the resulting low resting potential. This view was based on experiments with the locust, an herbivorous insect which, however, is anomalous in having blood ion ratios typical of carnivorous insects (Hoyle, 1955*c*). The presence of overshoots in *Carausius* muscle fibres suggests the possibility that some degree of adaptation to low resting potentials may be present in the process responsible for the active membrane response in herbivorous insects, though its probable basis is obscure. It is also possible that the coupling of contraction to membrane depolarization is not a direct one in insects: Hoyle (1957*b*) has presented evidence that in crustaceans it is not. A complex coupling mechanism could quite possibly possess properties which would allow adaptations resulting in increased activation of the contractile mechanism by smaller depolarization. The mechanical performance of *Carausius* muscle is of interest in this connexion. The tension developed by fibres of its prothoracic flexor tibialis is four-fifths of that developed by fibres of the locust jumping muscle (both

muscles in their natural media). Yet the action potential in *Carausius* is only a little over one-half that of the locust in size (see Hoyle, 1955c).

The very high tetanus/twitch ratio in *Carausius* lends support to the suggestion made by Hoyle (1955b) that in arthropods the contractile material is not fully activated by a single muscle depolarization, however complete, but that more than one is required for full activation.

The F response in *Carausius* is similar to that observed in other insects (see Hoyle, 1957a) and to certain 'fast' responses found in crustaceans by Furshpan (1955). Detailed studies of insect fast responses have been confined to the locust (del Castillo *et al.* 1955; Hoyle, 1955b, c, 1957a) and to a lesser extent to the cockroach (Hoyle, 1955c). In the locust, progressive lowering of the temperature results in a lengthening of the time course and a reduction in size of the response, and an inflexion becomes increasingly apparent in the rising phase. Below about 8° C. the active membrane response disappears. The F response in *Carausius* is affected by temperature variation in the same way, but to a lesser degree; for example, the active membrane response is still present at 5° C. This is surprising at first sight, because according to Chopard (1938) *C. morosus* is a strictly tropical insect from the Far East, where diurnal and seasonal temperatures remain at a fairly high and constant level. Some degree of accommodation may have resulted from continuous breeding in this country for many years.

The ability of the junctional potential to summate shows it to be a graded response. Summation could be due to the liberation of a further quantity of a transmitter substance by a subsequent stimulus. The active membrane response, on the other hand, exhibits refractoriness and does not appear to summate with a further active membrane response. This suggests an all-or-nothing process. In vertebrates Hodgkin (1951) has shown that refractoriness of the spike potential results from the high potassium conductance which occurs during this phase of the action potential, and to a lesser extent to the inactivation of the 'sodium carrier' by the intense depolarization of the propagated response. Wood (1957) obtained evidence that a specific 'sodium carrier' may not be present in *Carausius*, but that the process responsible for the production of the action potential could be explained on a similar general basis to that which produces the vertebrate action potential. The present results support this view. If it is correct, the nature of the neuromuscular transmitter is of interest. There is no direct evidence that a transmitter substance occurs in insects, but its presence in the locust and cockroach has been inferred by Hoyle (1955c). The lack of any action of common pharmacological drugs suggests that neuromuscular transmission in *Carausius* is not cholinergic, nor is there any indication that the transmitter is any other well-known drug. The effects of some quaternary ammonium compounds other than acetylcholine are ambiguous (Wood, 1957), but do not encourage the view that they are involved. However, it is just possible that the diffusion barrier set up by the tracheolated connective tissue around the muscle fibres of *Carausius* and other insects (Hoyle, 1953; Wood, 1957) could prevent an externally applied drug from reaching the junctional receptors in sufficient concentration to overcome the action of any destructive enzyme system

which might be present. But if this was so, physostigmine might still be expected to exert some effect. The fact that it has none strengthens the probability that acetylcholine is not the transmitter. The possibility of a diffusion barrier effect of this kind is lessened, but not disproved, by the lack of effect of hyaluronidase and trypsin.

The S responses of *Carausius* muscle fibres are comparable with the S_1 responses described by Hoyle (1955*b*) in the locust, and with the slow responses observed by Furshpan (1955) in the muscle fibres of *Cambarus*, although they more nearly resemble the latter in exhibiting no active membrane response. The alterations in speed and extent of contraction accompanying changes in the frequency of stimulation of the S axon show that very delicately graded movements of the muscle could be obtained naturally by the animal by this means. During spontaneous activity S responses have been observed which appear to hold the muscle at a particular level of contraction. There seems to be no need for a second slow response of the type described by Hoyle (1955*b*) which in the locust is responsible for the performance of very slow movements and for the maintenance of tonus. The flexor tibialis of *Carausius* is innervated by a number of F axons which evoke similar responses. Control of fast activity could therefore be achieved by the animal both by altering the frequency of impulses passing down each F axon and by the typical vertebrate method of varying the number of axons operating at a given time. There is no evidence that there may be a similar duplication of S axons, but if it occurs the control of slow activity would be even finer.

Friedrich (1933) claimed to have demonstrated peripheral inhibition in the flexor of *Carausius*. He stimulated the flexor at an intensity just below that required for excitation and observed a slight further relaxation of the resting muscle. He also stimulated the muscle at a similar intensity while it was relaxing from a contraction and found that the rate of relaxation increased. Ripley and Ewer (1951) suggested that this effect could have been obtained from a loosely held preparation by contraction of the coxal muscles. Another possibility is that reflex slow contraction of the extensor muscle occurred through stimulation of afferent fibres in the crural nerve. The effects, as shown in Friedrich's (1933) records, were in any case very slight. In the present work stimulation at different intensities, using the Kuffler & Vaughan Williams (1953) technique, has failed to reveal any evidence of the existence of an inhibitory axon; such histological evidence as there is, also argues against the presence of such an axon.

SUMMARY

1. The prothoracic flexor tibialis muscle of *Carausius morosus* consists of two lateral rows of pinnately arranged muscle units. Motor nerve endings of the 'Doyère-cone' type are distributed at intervals of approximately 60μ along each fibre. Each motor ending is probably innervated by two axons.
2. Two types of responses have been found in the muscle fibres: (i) 'fast' electrical responses resembling the action potential of vertebrate muscles, associated with twitch-type contractions of the fibres; (ii) 'slow' readily facilitating responses resembling end-plate potentials, associated with slow, smooth contractions of the

muscle, and with the maintenance of tonus. There is no evidence of peripheral motor inhibition.

3. The muscle bathed in haemolymph is capable of developing a tetanus tension of 800 g./cm.² cross-sectional area of individual muscle fibres. The tetanus:twitch ratio is over 25:1.

4. Pharmacological substances which affect excitable tissues of other animals have no effect on the fast response.

5. Progressively lowered temperatures lengthen the time course and reduce the amplitude of the fast response, but an active membrane response remains at 5° C.

6. Refractoriness is evident near the peak of the fast response. The junctional potentials will summate if sufficiently close in time.

7. It is suggested that the process underlying the fast response in *Carausius* is similar to that in the locust and in vertebrates; but neuromuscular transmission does not appear to be cholinergic.

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THE DIGESTION AND ABSORPTION OF TRIPALMITIN IN THE COCKROACH, *PERIPLANETA AMERICANA* L.

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(Received 30 June 1958)

INTRODUCTION

The digestion and absorption of fats in the cockroach has in the past been the subject of several extensive investigations. Plateau (1876), Sanford (1918), Swingle (1925), Abbott (1926), Wigglesworth (1928) and Schlottke (1937) have all made *in vitro* studies of fat hydrolysis with various species of cockroach. It is only relatively recently that an *in vivo* study has been made of the hydrolysis of some fats in the cockroach crop by Eisner (1955).

There has been considerable controversy as to the site of absorption of fats in these insects. Petrunkevitch (1900) demonstrated histologically an accumulation of fat droplets in the crop epithelium following a fat meal, and concluded that this organ was of major importance in fat absorption. Subsequently, Sanford (1918) and Abbott (1926) obtained similar results and attempted to dispose of the objections made to this hypothesis by Cuénot (1896), de Sinéty (1901) and Schlüter (1912). More recently, Eisner (1955) has made a detailed study of the factors involved in the appearance of fat droplets in the crop epithelium following fat meals. All these investigations have been qualitative in nature and no quantitative study has been made of fat absorption in the alimentary canal of the cockroach. In the present investigation an attempt has been made to follow the digestion and absorption of a ^{14}C -labelled triglyceride using the technique previously employed to follow the absorption of glucose in *Periplaneta americana* L. Tripalmitin was available suitably labelled with ^{14}C and was chosen for use in this investigation. The glyceryl esters of palmitic acid are among those most commonly occurring in natural fats (Hilditch, 1947).

METHODS

As in a previous investigation (Treherne, 1957*b*), adult cockroaches were starved for 7 days in order to obtain individuals in which the alimentary canal was empty. They were given access to water, except for 24 hr. immediately preceding the experiment. In each experiment the starved individual was allowed to consume 0.10 ml. of an experimental fluid. After an appropriate experimental period the insect was killed by immersion in boiling water. The whole of the alimentary canal was immediately removed and the contents of the various parts squeezed into calibrated centrifuge tubes using the tips of fine pairs of forceps.

The experimental fluid contained 0.2% ^{14}C -labelled tripalmitin (palmitate-1- C_{14}) which was suspended in a 2.0% solution of the inert cellulose ester 'Cellofas B' (sodium carboxymethylcellulose). This substance produced a fluid of treacle-like consistency. In some experiments the tripalmitin was dissolved in oleic acid, a 10.0% suspension being emulsified with 'Cellofas B'. The experimental fluid also contained 0.5 M/l. glucose and 0.008 M/l. of the dye Amaranth (Azo-Rubin S).

The gut contents were extracted in the centrifuge tubes with hot chloroform and, after cooling, the volume was adjusted to 1.0 ml. This solution was then pipetted off for assay of radioactivity and chromatographic analysis. 5.0 ml. of a solution buffered to pH 10.0 was then added to each tube to bring the dye into the solution. This was centrifuged at 4000 r.p.m. and the concentration of the dye in the supernatant was determined at pH 10.0, using a Unicam absorptiometer at an absorption maximum of 510 m μ . The radioactivity of the labelled material was assayed by determining the activity of measured volumes placed on lens papers (Reid, 1947), using a thin-windowed Geiger-Müller tube (G.E.C. CV 2139).

The dye Amaranth was used as a marker in these experiments, for it has been shown that it is not absorbed from the lumen of the cockroach gut (Treherne, 1957*b*). As in previous studies the dye has been used to measure transit in the gut and, by comparing the ratio of dye to radioactive substances in the various parts of the gut, to determine the net percentage absorption of tripalmitin and its derivatives.

The reverse-phase method of paper chromatography devised by Mangold, Lamp & Schlenk (1955) was used in an attempt to follow any hydrolysis of tripalmitin occurring in the gut lumen. For this purpose, Whatman No. 1 filter-paper was dried for at least 2 hr. at 120° C. and then drawn through a 5.0% solution of silicone fluid (M.S. 200/10 c.) dissolved in ether. 2.0 μl . samples of radioactive material dissolved in chloroform were applied to the base-line on these papers. The chromatograms were developed by descending chromatography using the solvent systems chloroform/methanol and tetrahydrofuran/water (Mangold *et al.* 1955). Samples of tripalmitin and its derivatives were run as markers on the chromatograms. Palmitic acid was detected by treating the paper with 1.0% copper acetate followed by 0.1% Rhodamine B according to the method of Savary (1954). Tri- and dipalmitin were hydrolysed on the paper by spraying with 1.0% pancreatin and incubating at 37.0° C. (Mangold *et al.* 1955), prior to the copper acetate treatment. Monopalmitin was detected by spraying the papers with a 1.0% solution of lead tetra-acetate in absolute benzene (Mangold *et al.* 1955). To assay the chromatograms for radioactivity the papers were cut into strips and placed over a 1.0 cm. wide slit in a piece of Perspex, beneath which was a thin-windowed Geiger-Müller tube. Counts were made on successive 1.0 cm. wide areas until the whole of the strip had been assayed.

All the insects were reared and the experiments carried out at a temperature of $28.0 \pm 1.0^\circ \text{C}$.

RESULTS

To investigate the extent of any hydrolysis of tripalmitin cockroaches were fed 0.1 ml. amounts of the experimental fluid containing ^{14}C -labelled tripalmitin. In one set of experiments 0.2% tripalmitin was suspended in the experimental fluid, in another the tripalmitin was dissolved in emulsified 10.0% oleic acid. The extent of the hydrolysis of 0.2% tripalmitin in oleic acid is illustrated in Fig. 1 for chromatograms developed with chloroform/methanol as the solvent system. At 2.0

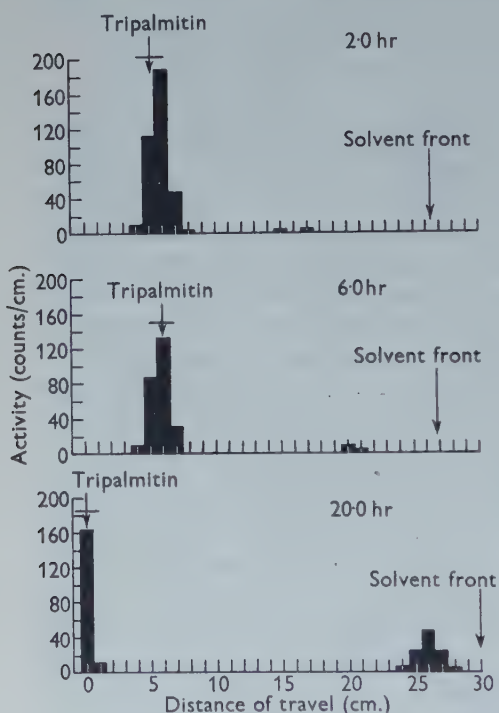


Fig. 1

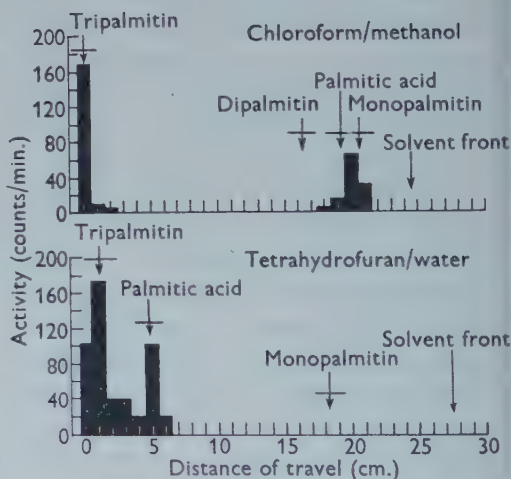


Fig. 2

Fig. 1. The distribution of radioactivity on paper chromatograms of the crop contents at various times after ingestion of ^{14}C -labelled tripalmitin. The solvent system used in these experiments was chloroform/methanol.

Fig. 2. The radioactivity on paper chromatograms of the crop contents, developed in two solvent systems, 20.0 hr. after the ingestion of ^{14}C -labelled tripalmitin. The symbols above the histograms represent the position and extent of control spots run adjacent to the experimental ones.

and 6.0 hr. after feeding most of the radioactivity was associated with the tripalmitin spot, which showed R_F values varying between 0 and 0.22. After 20 hr. a second, smaller, peak of radioactivity was obtained with an R_F value of 0.80–0.90. When control samples of tripalmitin, dipalmitin, monopalmitin and palmitic acid were run on chromatograms this second radioactive peak was associated with the palmitic acid spot in both solvent systems (Fig. 2). Substantially similar results were obtained

with tripalmitin suspended alone in the experimental fluid. The amount of radioactive material which could be recovered from the lumen of the mid-gut was not sufficient for a satisfactory chromatographic analysis.

Table 1. *The percentage of ^{14}C remaining incorporated as tripalmitin in the crop after an experimental period of 20.0 hr.*

Experiment	Serial	% tripalmitin	Mean \pm S.D.
0.2 % tripalmitin	1	80.0	77.4 \pm 10.1
	2	86.1	
	3	62.6	
	4	67.0	
	5	61.8	
	6	83.6	
	7	84.1	
	8	94.4	
0.2 % tripalmitin in oleic acid	1	63.6	77.7 \pm 11.7
	2	75.9	
	3	79.2	
	4	92.0	

Table 1 records the proportion of ^{14}C in the crop which remained incorporated as tripalmitin after 20.0 hr. It will be seen that there was considerable variation in the extent of the hydrolysis as between different individuals. These results show that whether administered alone or dissolved in oleic acid the amount of unhydrolysed tripalmitin remaining in the crop after 20.0 hr. averaged approximately 77 % of the total.

The absorption of the ^{14}C -labelled tripalmitin was followed in some experiments in which starved individuals were fed 0.1 ml. amounts of the experimental fluids. The net percentage absorption of the labelled compounds was calculated from the radioactive-material/dye ratio in the various parts of the alimentary canal. The absorption was determined in insects which had been fed either tripalmitin suspended in the experimental fluid or tripalmitin dissolved in emulsified oleic acid (Figs. 3, 4). In both cases little absorption appeared to take place from the crop, but there was a very rapid disappearance from the lumen when the fluid entered the mid-gut region. The results obtained after 2.0 hr. indicate that absorption must be largely confined to the caeca and the anterior part of the ventriculus. Unfortunately it was impossible to separate the parts played by these two regions of the mid-gut using the present technique as the experimental fluid always appeared in them simultaneously.

Table 2 records the net percentage absorption of ^{14}C -labelled tripalmitin and its derivatives from the crop after a period of 20.0 hr. The negative values recorded resulted from experiments in which the ^{14}C recovered from the crop apparently exceeded the amount of dye present, these figures falling within the normal experimental error of the method. The results show that under these experimental conditions any absorption from the crop must be very small, certainly less than about 4 %, which is within the experimental error of the method.

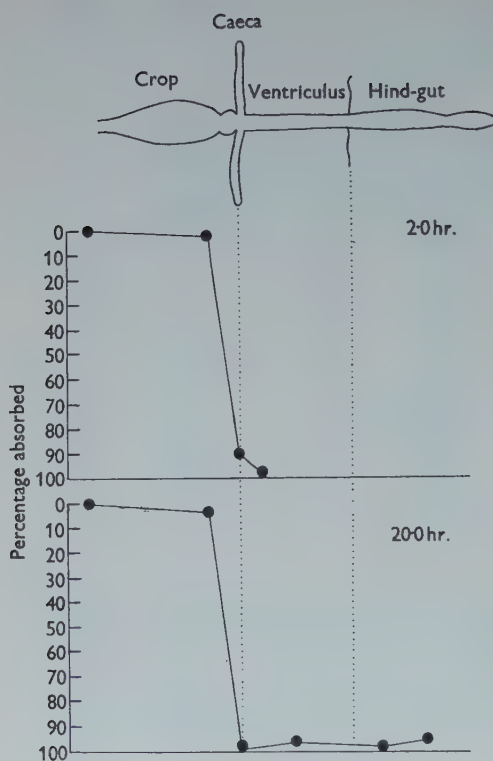


Fig. 3

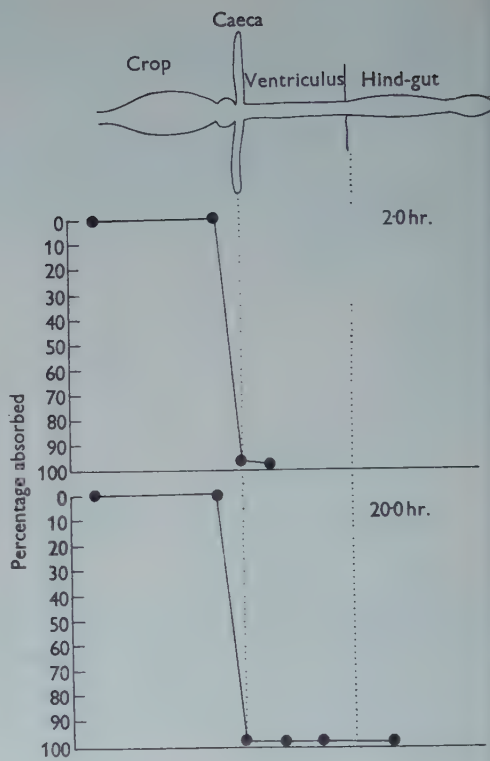


Fig. 4

Fig. 3. The percentage absorption of ^{14}C -labelled tripalmitin and its derivatives 2.0 and 20.0 hr. after ingestion of a suspension of 0.2 % tripalmitin.

Fig. 4. The percentage absorption of ^{14}C -labelled tripalmitin and its derivatives after the ingestion of 0.2 % tripalmitin dissolved in 10.0 % emulsified oleic acid.

Table 2. *The percentage absorption of ^{14}C -labelled material from the crop after an experimental period of 20.0 hr.*

Experiment	Serial	% absorbed	Mean \pm S.D.
0.2 % tripalmitin	1	1.6	0.6 \pm 3.25
	2	5.0	
	3	-3.9	
	4	3.3	
	5	-1.6	
	6	0.2	
0.2 % tripalmitin in oleic acid.	1	2.7	0.5 \pm 1.84
	2	-2.3	
	3	1.1	
	4	0.1	
	5	1.0	

It has been shown that the absorption of the radioactive material was largely confined to the anterior part of the mid-gut where the uptake was relatively rapid. This effect suggested that the rate at which the fluid was allowed to leave the crop was likely to be of importance in determining the total absorption of tripalmitin and its derivatives. Some experiments were therefore carried out in which the degree of crop emptying was compared with the total amount of radioactive material absorbed from the gut during the experimental period. The extent of the crop emptying was determined from the amount of dye present in the crop, while the absorption was calculated from the total amount of radioactive material recovered from the crop and the remainder of the alimentary canal. Fig. 5 illustrates the close relation which was obtained between crop emptying and the absorption of tripalmitin in the cockroach.

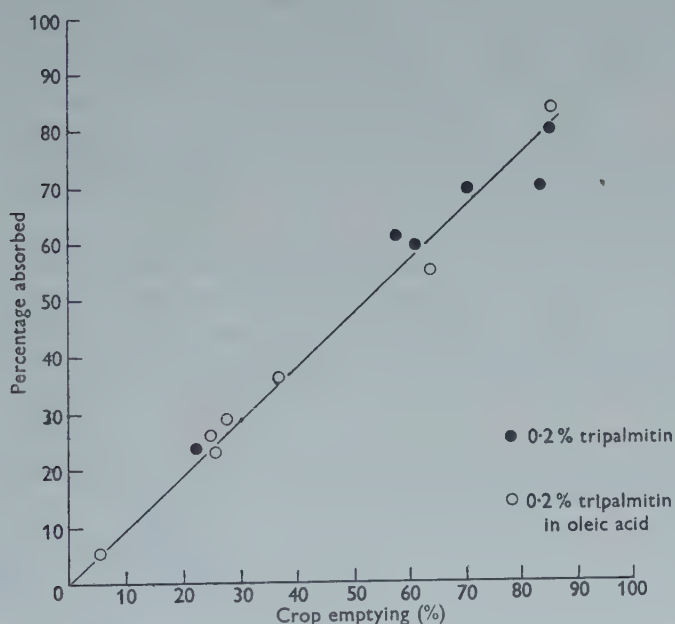


Fig. 5. The relation of crop emptying to the absorption of ^{14}C -labelled tripalmitin.

DISCUSSION

It is evident that under these experimental conditions there was a partial hydrolysis of tripalmitin in the crop. After 20.0 hr., when the crop had nearly emptied, about three-quarters of the triglyceride remained unhydrolysed. These observations are in agreement with those of Eisner (1955) who showed that the hydrolysis of triolein in *Periplaneta americana* did not proceed to completion. He argued that the completion of hydrolysis was prevented by the accumulation of the fatty acid within the partly hydrolysed fat. It was concluded that, as was shown by Frazer (1948), the enzyme was displaced by the end-products which accumulated at the oil/water interface on which the lipase acts. The degree of hydrolysis found for tripalmitin

in the present investigation is of the same order as that obtained by Frazer (1948) who found that hydrolysis stopped when about 30% of the triglyceride had been digested.

During the first few hours after the experimental meal there was no significant hydrolysis of tripalmitin in the crop and it passed into the mid-gut in a largely unhydrolysed condition. Now crop emptying, which is an exponential function of time in this insect (Treherne, 1957*b*), would tend to proceed most rapidly during this period immediately following the meal. These facts cannot, however, be taken as evidence for the absorption of triglycerides in the unhydrolysed condition, for it is possible that there was some further relatively rapid hydrolysis of the tripalmitin in the mid-gut where the enzyme/substrate ratio is likely to be much higher than in the crop lumen. It should be borne in mind in this respect that the main source of lipase in the cockroach gut is the epithelium of the mid-gut (Abbott, 1926).

The results for the degree of hydrolysis of tripalmitin in the crop were characterized by considerable individual variation. This perhaps results from the mode of production of lipase in the crop, for it is secreted in the mid-gut and passes forward to mix with the crop contents (Abbott, 1926). It seems likely with such a system that the appearance of the enzyme in the crop may be determined by several factors which together might result in a certain degree of variability in the hydrolysis of ingested triglycerides.

It has already been mentioned that some controversy has existed in the past about the part played by the crop in fat absorption in the cockroach. The hypothesis that the crop was of importance in fat absorption was based on the histological observations of Petrunkevitch (1900), Sanford (1918) and Abbott (1926) that fat droplets appeared in the epithelium of the crop following a meal containing fat. More recently, Eisner (1955) examined this process in some detail and showed qualitatively that the appearance of fat droplets in the epithelium depended on the viscosity of the fat. The accumulation of fatty acids in the partly hydrolysed long-chain triglycerides lowered the viscosity and hastened the appearance of fat droplets in the crop epithelium. Eisner found that heavy mineral oils could be made to appear quite rapidly when mixed with oleic acid. Fat droplets appeared in the epithelium when olive oil was fed to the insects, alone or as an aqueous emulsion.

The results obtained in the present investigation do not support the hypothesis that the crop is an important organ in fat absorption. The experiments carried out on the absorption of tripalmitin and its derivatives demonstrated that under these experimental conditions there was no significant uptake from the crop. These substances were, however, absorbed extremely rapidly from the lumen of the caeca and the anterior part of the ventriculus. It may be that the absorption of tripalmitin in the crop may differ from that of a substance such as olive oil used by these authors. However, from the observations of Eisner (1955) it would certainly be expected that tripalmitin dissolved in oleic acid would be absorbed in the crop, for he showed that the absorption of oil mixtures seemed to depend largely on their viscosity. The fact that it was not absorbed in significant amounts in the present investigation suggests that the droplets of fat observed in the epithelium represented

only a very small proportion of the total fat, the greater part of which was absorbed in the mid-gut region. Furthermore, it should be borne in mind that in these experiments the tripalmitin was administered to the insect suspended in 0.5 M/l. glucose in order to slow down crop emptying (Treherne, 1957*b*) and any absorption from the crop would thus be exaggerated. Wigglesworth (1942) has shown that droplets of oil can be made to appear in the cuticular epithelium of *Rhodnius* when oleic acid or olive oil is placed on the abdominal surface. There is therefore no reason to suppose that the crop wall of the cockroach is any more permeable to fats than the rest of the body surface.

This account does not supply any information about the absorption of the glycerol released on the hydrolysis of the tripalmitin, for this portion of the molecule was not labelled with ^{14}C . It seems unlikely, however, that appreciable amounts of glycerol would be absorbed in the crop. The crop is lined with a cuticular wax layer which is relatively impermeable to water (Eidmann, 1922) and, by analogy with other water-soluble compounds (Treherne, 1957*a*), is likely to provide a relatively impermeable barrier to the diffusion of glycerol.

The total amount of ^{14}C -labelled material absorbed showed a linear relation with crop emptying. Thus, as with glucose (Treherne, 1957*b*), the limiting process in absorption was not the transfer of material across the gut wall, but the rate at which it was allowed to leave the crop. This system effectively masks the processes at the site of absorption and the present investigation does not, therefore, throw any light on the mechanism of uptake in the mid-gut. It is possible that these processes may be better studied in a preparation in which the mid-gut can be filled with a fluid of known concentration, as has been described in some previous investigations (Treherne, 1958*a, b*).

SUMMARY

1. A partial hydrolysis of ^{14}C -labelled tripalmitin has been demonstrated in the crop of *Periplaneta americana* L.
2. No significant absorption of tripalmitin and its derivatives could be demonstrated in the crop, whether the tripalmitin was suspended in an experimental fluid or dissolved in emulsified oleic acid.
3. Absorption took place in the mid-gut and appeared to be largely confined to the caeca and the anterior part of the ventriculus.
4. The total absorption of tripalmitin showed a linear relation with crop emptying, suggesting that the rate at which the material was allowed to leave the crop, rather than the uptake in the mid-gut, was the limiting factor in absorption.

I am grateful to Dr F. Aylward (Department of Chemistry and Food Technology, Borough Polytechnic), Mr B. J. F. Hudson (Unilever Ltd.), Dr J. Jasperson (Bibby and Sons Ltd.) and Dr T. Malkin (Department of Organic Chemistry, Bristol University) for their generous gifts of samples of various partial glycerides.

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EXCRETION BY THE MALPIGHIAN TUBULES OF THE STICK INSECT, *DIXIPPUS MOROSUS* (ORTHOPTERA, PHASMIDAE): AMINO ACIDS, SUGARS AND UREA

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(Received 19 July 1958)

INTRODUCTION

In a series of papers (Ramsay, 1954, 1955*a, b*, 1956) the author has made a study of the excretion of inorganic ions by the Malpighian tubules of the stick insect. The only ion which is undoubtedly actively transported from haemolymph to urine under normal conditions is potassium, and on the basis of this fact it has been suggested that the secretion of potassium is, as it were, the prime mover in the process or urine formation, water and other ions following passively. It now becomes of interest to study the passage of organic molecules across the wall of the tubule. For organic substances information is available only in the case of uric acid, for which it is known that in the stick insect the concentration in the urine is greater than in the haemolymph (Ramsay, 1955*a*) and in respect of which an excretory mechanism has been proposed by Wigglesworth (1931) for *Rhodnius*.

Where the primary process of urine formation is ultrafiltration, as in the normal vertebrate nephron, the glomerular urine has the same composition as an ultrafiltrate of the blood and one would expect all solutes of low molecular weight present in the blood to appear in the urine unless they are specifically reabsorbed in the tubule. But in an aglomerular nephron, in which urine is formed by a process of secretion, one would not expect to find substances in the urine unless they are specifically excreted, and one would not expect to find excretory mechanisms specifically evolved in respect of metabolically useful constituents of the blood.

The Malpighian tubules of insects appear to have more in common with aglomerular than with glomerular nephrons of vertebrates, and for this reason one would not expect to find that they allow metabolically useful substances to pass into the urine. But, to anticipate, it is found that they do. One must therefore be prepared to readjust one's first conceptions of the role of the Malpighian tubules in the insect's physiological economy.

PRELIMINARY EXPERIMENTS

Very little is known about the uptake and translocation, let alone excretion, of organic substances in insects. It was therefore considered necessary to undertake certain preliminary investigations. The method used was a simple form of one-dimensional paper chromatography, with development in 80% propanol, and it is

to be understood that 'amino acids' refers to substances reacting with ninhydrin and that 'sugars' refers to substances reacting with aniline phthalate.

Gut contents. Amino acids were detectable in the fore-gut, present in quantity in the mid-gut and absent from the hind-gut. The contents of the fore-gut and mid-gut, but not of the hind-gut, gave a positive reaction with aniline phthalate, but separate spots were not recognizable on the chromatogram. After massive injection into the haemolymph glucose, but not glycine, was detectable in the faeces.

Gut exudate. Several experiments were carried out in which the gut of a well-fed insect was dissected out, tied off at mouth and anus and allowed to remain for some hours in a small volume of Ringer (for composition, see Ramsay, 1955*b*). The Ringer was then examined by chromatography. In some cases the fore-, mid- and hind-gut were tied off separately and studied in isolation.

Amino acids were easily detectable in the Ringer surrounding the whole gut, and when separate regions were studied it was found that nearly all the amino acids came from the mid-gut. In further experiments the separate regions of the gut were filled with Ringer containing 70 mM./l. glycine (made up with indian ink to reveal any gross perforation of the gut wall) and similarly tested. It was found that there was slightly more output of glycine from the mid-gut than from the fore-gut; the greatest output was from the hind-gut and was associated with an obvious decrease in the volume of the hind-gut contents. Glucose was similarly reabsorbed from the hind-gut.

Haemolymph. The haemolymph has a relatively high concentration of protein and peptides and easily detectable concentrations of such amino acids as alanine, glycine and valine. The amino acid pattern as seen in chromatograms from different insects is by no means constant, and it might be thought that the pattern corresponded to a steady state between absorption from the gut and assimilation by the tissues. To some extent this is true. In the haemolymph taken from an insect which has starved for a week alanine and valine are undetectable, but glycine and other amino acids as yet unidentified are scarcely diminished in concentration. The amino acids of the haemolymph are apparently maintained in balance with the proteins of the haemolymph and of the tissues, while also being affected by current absorption from the gut.

After the preliminary investigations had been completed Treherne (1958) showed that in the locust ^{14}C -labelled glucose absorbed from the gut appears in the haemolymph as the disaccharide trehalose, originally reported in insect blood by Wyatt & Kalf (1956). This matter was then investigated on the stick insect and the haemolymph was found to contain a substance which corresponded with trehalose on the chromatogram. ^{14}C -labelled glucose was injected into the haemolymph, which was collected about 12 hr. later, and it was found that practically all the activity was concentrated at the trehalose spot. The inadequate chemical characterization of trehalose in these preliminary investigations makes it impossible to speak with certainty; but it seems likely that rapid conversion of glucose to trehalose occurs in the stick insect as in the locust.

Urine. Urine was obtained from Malpighian tubules isolated singly or in pairs,

in drops of haemolymph as described elsewhere (Ramsay, 1954). Normal urine contains at least one amino acid but this has not been identified, nor its presence in haemolymph confirmed. Glycine, although present in haemolymph at a concentration of about 10 mm./l., is not detectable in normal urine by chromatography, but it can be detected in the urine if its concentration in the haemolymph is raised to about 70 mm./l. Glucose is not detectable even in haemolymph, but when added to haemolymph to give a concentration of about 70 mm./l. it is readily detectable in the urine. Trehalose is not detectable in the urine by chromatography.

These preliminary investigations indicate the following general conclusions, which must of course be regarded as provisional.

Sugars such as glucose are so rapidly metabolized that their concentrations in the haemolymph are normally very low; they are not presented to the excretory system in measurable concentration. When its concentration in the haemolymph is artificially increased, glucose is fairly rapidly excreted by the Malpighian tubules. Some of this glucose leaves the body with the faeces but most of it is reabsorbed from the hind gut.

Amino acids are present in the haemolymph in varying concentrations which in some cases can be related to the state of digestion. Most of these are not detectable in the urine, but glycine appears in the urine if its concentration in the haemolymph is raised. Like glucose it is reabsorbed from the hind-gut.

It therefore appears that, contrary to expectation, metabolically useful substances can pass into the urine; and this at once raises further questions. Is glycine, for example, normally present in urine but at a concentration too low to be detected by simple methods, or is its appearance in the urine a 'threshold' phenomenon related to a certain critical concentration in the haemolymph? Does the tubule simply allow a certain proportion of the glycine in the haemolymph to escape or does it act in such a way as to regulate the concentration of this substance in the haemolymph?

Answers to these and other questions are sought in the work now to be described.

MATERIAL AND METHODS

All the experiments herein to be described were carried out on the 'superior' tubules (de Sinéty, 1901). These were mounted as described earlier either singly or in pairs in small drops (about 25 μ l. volume*) of medium under liquid paraffin. Collection was continued until sufficient volume of urine had been produced; after 12 hr. volumes of the order of 1 μ l. were obtainable.

Although quantitative chemical techniques have been worked out for most of the substances studied in the present work, these techniques are generally more laborious than techniques involving the use of radioactive labelling. Many ^{14}C -labelled organic compounds of physiological interest are now available but at such cost that some restriction must be placed upon full freedom of choice. From among the available amino acids six were selected—alanine, arginine, glycine, lysine, proline

* In earlier papers volumes have been given in 'mm.³' and 'mm.³ $\times 10^{-3}$ '. In the present paper these measures will be written ' μ l.' and 'm μ l.' respectively.

and valine—largely on account of their high solubility in water which made it possible to test them over a wide range of concentration. From among the carbohydrates, glucose, fructose and sucrose were selected. In addition to these two classes of compound urea was also included in the investigation.

If it is desired to obtain a relatively high concentration of the organic substance in the medium without at the same time raising the osmotic pressure it follows that the other constituents of the medium must be correspondingly diluted. The possibility therefore exists that observed effects upon the secretion of urine, which might be attributed to the presence of the specified organic substance, could equally be due to the dilution of some other constituent normally present. This consideration applies with special force to potassium, the rate of urine flow being greatly affected by the concentration of this ion in the medium. For this reason the concentration of potassium was kept approximately constant in the various media used (except where stated otherwise) and the other constituents were allowed to suffer dilution.

The standard method of making up media can be most easily described by taking a particular example. The Ringer used for making up media differed from the usual mixture in that no sucrose was added; the other constituents were uniformly concentrated so as to give a freezing-point depression of $\Delta = 0.55^\circ \text{C}$. and a potassium concentration of 21 m.equiv./l. (solution A). 1.06 mg. of ^{14}C -alanine, having an activity of 0.05 mc., was dissolved in 1 ml. of solution A, giving a concentration of alanine of approximately 12 mm./l. (solution B). Solution C contained 262 mm./l. of carrier alanine and 20 mm./l. KCl, and had a freezing-point depression of $\Delta = 0.56^\circ \text{C}$. The stick insect serum used in this work contained approximately 10 mm./l. alanine and 18 m.equiv./l. potassium, and will be referred to as solution D. These solutions were mixed as shown in Table 1.

Table 1

Solution A: Ringer; K, 21 m.equiv./l.
 B: Ringer; K, 21 m.equiv./l., ^{14}C -alanine, 12 mm./l.
 C: Carrier; K, 20 m.equiv./l., alanine, 262 mm./l.
 D: Serum; K, 18 m.equiv./l., alanine, 10 mm./l.

Medium	A (ml.)	B (ml.)	C (ml.)	D (ml.)	Nominal concentrations	
					Alanine (mm./l.)	Potassium (m.equiv./l.)
I	0.3	0.1	0.0	0.1	4.4	20
II	0.2	0.1	0.1	0.1	56	20
III	0.1	0.1	0.2	0.1	109	20
IV	0.0	0.1	0.3	0.1	161	20

In this way four media were prepared having different concentrations of alanine but having the same concentration of potassium and the same activity, this latter being the lowest activity convenient for conventional counting procedures with the volumes of urine available.

For experiments of 24 hr. duration it was found necessary to add an antibiotic to the medium, and for this terramycin was used, penicillin having been found

to be ineffective. The pH of all media was adjusted to 6.7 by addition of NaOH and was checked at the beginning of each experiment.

The design of experiments—and, even more, their interpretation—is complicated by the fact that many organic substances are metabolized as they pass through the tubule. When chromatographic separation was performed on the urine it was sometimes found that the activity was not exclusively associated with the spot given by the labelled substance. This was more noticeable, as one would expect, when the labelled substance was present in low concentration. When the medium was tested in the same way the activity was found to be confined to the area of the spot, which is again as one would expect, since the total amount of labelled substance present in the medium was very much greater than the total amount present in the urine and a smaller proportion of the amount in the medium—if, indeed, any—would be metabolized. Nevertheless, some doubt was felt about this interpretation since in chromatograms of urine the activity not associated with the spot was sometimes rather diffusely spread as if something was present in urine which interfered with the normal running of the chromatogram; and this, rather than metabolization, might account for the spread of the activity.

To put this to the test the following experiment was carried out using valine. Eight tubules were set up in a medium (*X*) containing labelled valine in low concentration but high specific activity and eight tubules were set up in normal serum (*Y*). Urine collected from *X* and from *Y* were pooled separately. To urine *X* inactive carrier valine was added. To urine *Y* inactive carrier valine and labelled valine were added to bring the total concentration and the activity to approximately the same values as in *X*. *X* and *Y* prepared in this way both contained labelled valine; but in *X* the labelled valine had passed through the tubule, whereas in *Y* it had not. If the spread of activity on the chromatogram were due to interference it should occur equally in *X* and *Y*, but if due to metabolization it should appear in *Y* only. In the event 74% of the total activity in *X* was concentrated at the valine spot, and 97% in *Y*. It was therefore concluded that the spread of activity in *X* could not be attributed to interference from substances present in urine but must be due to metabolization of the labelled valine during this passage through the tubule.

It is possible to assess the excretion of a labelled substance in two ways: (1) by counting the urine directly, which will give the total activity and a measure of the amount of labelled substance removed from the medium: and (2) by performing a chromatographic separation on the urine and then counting the activity associated with the appropriate spot, which will give a measure of the amount of labelled substance finally present in the urine. For most organic substances studied in this investigation both methods were used, and of course where mixtures were involved only the second method was practicable. Chromatograms were developed with 80% propanol–water or with butanol–acetic acid. The strips were first scanned under the counter and the appropriate active areas were cut out; each area was then eluted and eluate was evaporated to dryness on a counting planchette. In all experiments the count rate of a certain volume of urine was compared with the count rate of

the same volume of medium and, following the convention of vertebrate physiology, was expressed as the U/P ratio for the substance in question, where

$$\frac{U}{P} = \frac{\text{count rate of urine}}{\text{count rate of medium}}.$$

By reason of the cost of ^{14}C -labelled compounds the experiments were planned to give the lowest counting rates consistent with the required accuracy. With the direct method (i.e. not involving chromatographic separation) relatively small samples were taken with a pipette of approximately $0.35\ \mu\text{l}$. capacity and were transferred directly to counting planchettes, evaporated to dryness and counted. The error of the whole process of sampling and counting was found to be of the order of $\pm 7\%$ (standard deviation as % of the mean). Since the corresponding figure for the variation between urine samples taken under identical experimental conditions was of the order of $\pm 15\%$ the analytical error was considered acceptable. In these experiments the rate of urine flow was also recorded, the volume of urine produced being found by measuring the diameter of the droplet while it was allowed to sink through liquid paraffin. The error of this method is within $\pm 10\%$ of volume, which is again acceptable in view of variations in the observed rates of flow. When chromatographic separation, involving scanning of the paper, was carried out it was necessary to use larger quantities. Eight tubules were set up in each medium and the urine collected over a period of about 12 hr. was pooled. Samples of urine and medium were taken with a pipette of approximately $5\ \mu\text{l}$. capacity. When the active areas were subsequently eluted and counted the count rates were of course much higher and the counting error correspondingly reduced. It is estimated that the total analytical error in these determinations was of the order of $\pm 4\%$.

RESULTS

Amino acids

In the first series of experiments the direct method was used. The results of these experiments are summarized in Table 2, upon which the following comments may be made.

(i) All these substances appear in the urine; since they are all to be regarded as metabolically useful, this is in itself mildly surprising.

(ii) The U/P ratio is virtually independent of the concentration of the substance in the medium; this statement will appear to conflict with the evidence in Table 2 and must receive further explanation. The figures show that for all the amino acids (except lysine, for which no urine was produced at the higher concentrations) there is a tendency for the U/P ratio to increase with increasing concentration in the medium; this tendency is statistically highly significant ($P < 0.01$) for alanine, glycine and proline and is not significant ($P > 0.05$) for arginine and valine. But the increase in the U/P ratio is in all cases very small compared with the increase in the amino acid concentration in the medium. In the case of glycine the range of concentration in the medium is represented by a factor of 37, whereas the range of the U/P ratio is represented by a factor of 3.2; the factor for the medium is more than

Table 2

(Mean \pm standard deviation (no. of observations). Amino acids as specified in catalogue of Radiochemical Centre, Amersham. P , concentration in medium, mM./l.; U , concentration in urine, mM./l.; R , rate of urine flow, μ l./min.)

Amino acid	P	U/P	R
DL-Alanine-1- ^{14}C	4.4	0.23 ± 0.029 (9)	$1.69, 0.24$ (9)
	56	0.25 ± 0.048 (10)	1.45 ± 0.41 (10)
	109	0.32 ± 0.041 (6)	1.11 ± 0.43 (6)
	161	0.37 ± 0.061 (9)	1.19 ± 0.30 (9)
L-Arginine- ^{14}C (G)	0.34	0.39 ± 0.07 (4)	1.34 ± 0.14 (4)
	53	0.35 ± 0.09 (4)	1.29 ± 0.21 (4)
	105	0.40 ± 0.19 (4)	1.09 ± 0.29 (4)
	158	0.62 ± 0.29 (4)	0.40 ± 0.01 (4)
Glycine- ^{14}C (G)	4.5	0.14 ± 0.03 (4)	Not recorded
	43	0.17 ± 0.05 (7)	Not recorded
	82	0.22 ± 0.03 (6)	Not recorded
	121	0.27 ± 0.05 (5)	Not recorded
	166	0.45 ± 0.07 (4)	Not recorded
L-Lysine- ^{14}C (G)	0.15	0.13 (1)	Not recorded
L-Proline- ^{14}C (G)	0.16	0.22 ± 0.03 (4)	1.22 ± 0.54 (4)
	26	0.55 ± 0.08 (4)	2.25 ± 0.07 (4)
	52	0.59 ± 0.04 (4)	1.43 ± 0.54 (4)
	105	0.58 ± 0.13 (7)	1.94 ± 0.57 (7)
	157	0.63 ± 0.09 (4)	1.21 ± 0.75 (4)
DL-Valine-1- ^{14}C	6.3	0.24 ± 0.04 (8)	2.72 ± 0.42 (8)
	59	0.24 ± 0.04 (8)	2.36 ± 0.64 (7)
	111	0.23 ± 0.04 (8)	2.44 ± 0.61 (8)
	163	0.25 ± 0.05 (8)	2.40 ± 0.53 (7)

10 times greater than the factor for the U/P ratio. In all the other cases the contrast is even greater. When it is remembered that in order to achieve the highest concentrations of amino acid it has been necessary drastically to lower the concentrations of other constituents of the medium it would seem unlikely that the apparent increase in the U/P ratio is attributable to a direct effect of the concentration of the amino acid in the medium. The more striking fact is that not withstanding the very high and unphysiological concentrations which have been used one does not seem to have approached any limit to the capacity of the tubule to remove the amino acid from the medium—there appears to be no T_m value such as can be found for the transport of substances by the vertebrate nephron. One must also conclude that there is no evidence for any 'threshold' concentration below which these substances are not excreted and no evidence that the tubule acts so as to regulate their concentrations in the haemolymph.

(iii) The U/P ratios are different for the different amino acids being notably higher for proline than for the others.

(iv) There is a tendency for the rate of urine flow to decrease as the concentration of the amino acid in the medium increases. This tendency is statistically significant in the case of alanine and arginine, but not in the case of proline and valine.

The second series of experiments was designed to reveal any competition or other interaction between amino acids when presented to the tubule in a medium

containing three amino acids. Two such mixtures were made up, having regard to ease with which the components could be separated on a chromatogram; these were (a) arginine, proline and valine, and (b) alanine, glycine and valine. The mixtures were made from equal volumes of type IV media and thus contained each of the amino acids in 50–56 mm./l. concentrations. As controls the same three amino acids were tested in type II media in which they were present alone and in the same range of concentration. In each experiment four drops of each of the four media (mixture and three separates) were used and two tubules were set up in each drop, thirty-two tubules in all. Urines from all eight tubules in each medium were pooled and aliquots of 5 μ l. were spotted on paper, developed, eluted, dried and counted as described under Methods. The results of these experiments are given in Table 3. From these results it appears that the U/P ratios for the amino acids

Table 3

		U/P (mixture)	U/P (separate)
Group 1.	Arginine	0.30	0.25
	Proline	0.76	0.56
	Valine	0.29	0.20
2.	Alanine	0.22	0.24
	Glycine	0.32	0.19
	Valine	0.24	0.21

presented separately are very much the same as those seen in Table 2. In the case of the mixtures the U/P ratios are in general higher, notably in the case of glycine and proline. While this may be taken as evidence of interaction it may equally be related to the observation discussed above, namely, that the U/P ratio tends to increase with increasing amino acid concentration in the medium. There is certainly no evidence of competitive inhibition affecting the excretion of one amino acid in the presence of another.

Since for all the amino acids here studied the concentration in the urine is less than that in the medium, it is unnecessary to postulate active transport of the unionized molecules. At pH 6.7 alanine, glycine, proline and valine are on the alkaline side of their iso-electric points and therefore will be negatively charged; since the interior of the tubule is positive with respect to the exterior (Ramsay, 1955*b*) it follows, *a fortiori*, that it is unnecessary to postulate active transport of the ions. The reverse is true of arginine, and from a purely thermodynamic stand point its transport may be active; but one may note that its U/P ratio is not widely different from the U/P ratios of alanine, glycine and valine, which suggests that the same type of transport mechanism is operating in all cases.

If we are to take the view that all these amino acids are transported independently, each by its own specific transport mechanism, then we must concede that these transport mechanisms have capacities far in excess of those which are normally exercised in the body of the insect. The simpler view is that amino acids enter the urine by a passive, non-specific process.

Sugars and urea

The sugars tested were glucose, fructose and sucrose, and the experiments were carried out on the same lines as those just described for amino acids. The results obtained with single sugars are presented in Table 4, and the results obtained with urea are included in this table for convenience. Once again it is seen that all these substances appear in the urine. For the sugars the U/P ratios at the lowest values of P are significantly lower than at the three higher concentrations and this discrepancy is tentatively ascribed to metabolization. For glucose and fructose the U/P ratio does not change significantly with concentration in the range 53–158 mM./l. In the case of sucrose there is a small but significant increase in the U/P ratio over the same range as with certain amino acids. In the case of urea at all concentrations the U/P ratio does not differ significantly from 1, which is not surprising in view of the relative ease with which this substance is known to penetrate animal cells.

Table 4

(Mean \pm standard deviation (no. of observations). Labelled compounds as specified in catalogue of Radiochemical Centre, Amersham. P , concentration in medium, ml./l.; U , concentration in urine, mM./l.; R , rate of urine flow, $\text{m}\mu\text{l./min.}$)

Labelled Compound	P	U/P	R
D-Glucose (G)	0.17	0.32 ± 0.05 (5)	1.34, 1.46
	53	0.86 ± 0.18 (6)	2.04 ± 1.29 (4)
	105	0.90 ± 0.14 (6)	2.14 ± 1.04 (4)
	158	0.85 ± 0.07 (6)	1.59 ± 0.73 (4)
D-Fructose (G)	0.18	0.45 ± 0.06 (3)	1.50 ± 0.09 (3)
	53	0.61 ± 0.08 (3)	1.15 ± 0.47 (3)
	105	0.65 ± 0.07 (3)	0.87 ± 0.26 (3)
	158	0.56 ± 0.07 (3)	0.91 ± 0.47 (3)
Sucrose (G)	0.15	0.47 ± 0.07 (6)	2.63 ± 0.59 (6)
	53	0.53 ± 0.10 (6)	2.86 ± 0.52 (6)
	105	0.64 ± 0.03 (4)	2.08 ± 0.20 (4)
	158	0.71 ± 0.06 (5)	1.63 ± 0.09 (3)
Urea	3.1	1.03 ± 0.06 (6)	3.11 ± 1.90 (6)
	56	0.96 ± 0.06 (5)	3.95 ± 0.88 (5)
	108	0.99 ± 0.07 (6)	5.09 ± 2.04 (5)
	160	0.99 ± 0.03 (6)	5.62 ± 2.56 (6)

Experiments were also carried out with mixtures of glycine, glucose and urea, each substance being present in concentration of the order of 50 mM./l. The U/P ratios found in these experiments are presented in Table 5 together with the U/P ratios for the same substances when present alone. These latter figures are taken from Tables 2 and 4; in this part of the work the somewhat arduous task of simultaneous comparison, involving the rapid preparation of thirty-two tubules, was not attempted. Once again, the U/P ratio for each substance in the mixture is of the same order as the U/P ratio for the same substance alone. Urea appears to stimulate an increased rate of urine flow.

The general conclusions reached for amino acids appear to be equally applicable to the substances considered in the present section.

Table 5

(Mean \pm standard deviation (no. of observations). The figures for U/P (separate) are taken from Tables 2 and 4, using values of P nearest to 50 mm./l.)

	U/P (mixture)	U/P (separate)
Glycine	0.24 ± 0.04 (3)	0.17 ± 0.05 (7)
Glucose	0.95 ± 0.10 (4)	0.86 ± 0.18 (6)
Urea	0.92 ± 0.09 (4)	0.96 ± 0.06 (5)

EXCRETION AS A PROCESS OF DIFFUSION

For all the substances here investigated it has been found that the U/P ratio remains fairly constant and independent of the value of P . This is to be expected if these substances enter the tubule by passive diffusion.

If a simple process of diffusion is at work these substances should be able to pass equally easily through the wall of the tubule in the reverse direction. Rapid diffusion in the reverse direction has been demonstrated for urea and for sucrose in experiments of the following type. The main part of the tubule was placed in a drop

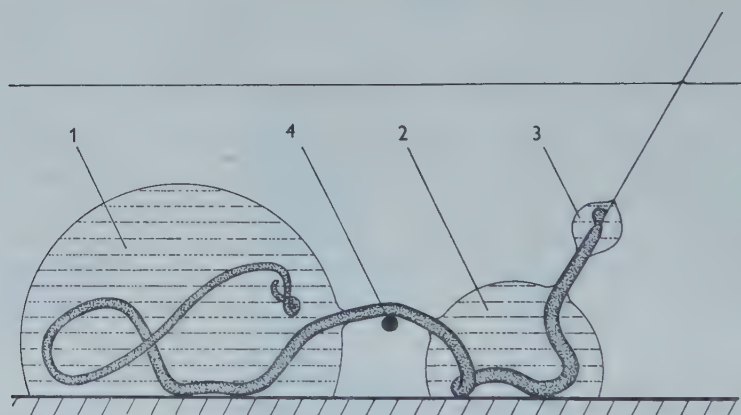


Fig. 1. Arrangement for demonstrating permeability of tubule in both directions. 1, large drop of medium containing labelled substance; 2, small drop of medium from which substance initially absent; 3, droplet of urine; 4, platinum wire support. All under liquid paraffin.

of medium containing the labelled substance, and the proximal end was arranged to pass through a second drop of medium in which the substance was not present (Fig. 1). After about 4 hr. the urine and the second drop of medium were collected entire and counted. From the results:

	Count of second drop (c.p.m.)	Count of urine (c.p.m.)
Urea	758	40
	691	39
Sucrose	524	17

it is obvious that most of the substance which might have been excreted in the urine has passed back through the wall of the tubule into the second drop. Both substances therefore can pass the tubule wall in both directions, although it has not been established that their rates in the two directions are the same. The other substances with which this paper is concerned were not tested.

The argument for the diffusion theory may be further tested by examining the effects of alterations in the rate of urine flow upon the U/P ratio. The rate of urine flow may be altered either (i) by altering the rate at which natural urine is formed by secretion through the walls of the tubule, or (ii) by perfusing the tubule with artificial urine. As will now be shown, the relationship between rate of flow and U/P ratio is different in the two cases.

We will represent the tubule as a tube of length L and radius r , closed at one end, and we will regard the wall of the tubule as a membrane of negligible thickness. We will suppose that water is actively pumped through unit area of wall into the lumen at a rate denoted by a . If R is the rate at which urine issues from the tubule, $R = 2\pi rLa$.

We will further suppose that a substance dissolved in the plasma at concentration P diffuses into the lumen of the tubule where it attains concentration U . The rate of diffusion will be proportional to the area of the wall of the tubule and to the concentration difference. If X is the quantity of the substance which enters the tubule in unit time, we have

$$X = b \cdot 2\pi rL(P - U),$$

where b is constant. The concentration of the substance in the urine is

$$U = \frac{X}{R} = \frac{X}{2\pi rLa}.$$

Substituting for X we obtain

$$\frac{U}{P} = \frac{b}{a + b}. \quad (1)$$

Equation (1) shows that the U/P ratio is independent of P , as the experimental results have indicated. Since the same relationship could have been derived by considering an element of the tubule wall instead of the whole tubule it follows that U has the same value at all points in the lumen of the tubule and that the speed with which the stream of urine passes along the lumen is irrelevant. This equation defines the effect upon the U/P ratio of alterations in the rate of secretion of natural urine.

We will now consider a tubule which is perfused with artificial urine at a rate v and will first seek to establish the time taken for a particle of urine to traverse the length of the tubule. For this purpose we will assume that over any cross-section the speed is uniform. If the tubule is not secreting natural urine then the speed of the stream is

$$\frac{dl}{dt} = \frac{v}{\pi r^2},$$

and the time taken to traverse the tubule is

$$t = \frac{\pi r^2 L}{v}.$$

But if the tubule is secreting urine then over a distance l from the point of injection a volume of natural urine equal to $2\pi r l a$ will be added to the artificial urine. The speed of the stream as it passes the cross-section at l is

$$\frac{dl}{dt} = \frac{v + 2\pi r l a}{\pi r^2},$$

and the time taken to traverse the tubule is

$$t' = \pi r^2 \frac{1}{2\pi r a} \log_e \left(\frac{v + 2\pi r L a}{v} \right).$$

As v becomes very much greater than $2\pi r L a$, t' approximates to t and it can be calculated that if $v = 10 \times 2\pi r L a$ then $(t - t')/t \times 100 = 4.8\%$, an error which may be accepted in relation to the errors of observation.

We will therefore assume that, as a result of perfusion, elements of volume traverse the tubule in time $t = \pi r^2 L/v$, during which time the substance diffuses into them. The same results would follow if the tubule were instantaneously filled with perfusion fluid, left for time t and then instantaneously emptied. The rate of entry of the substance will be proportional to the area of the wall of the tubule and to the concentration difference, so that

$$X = b \cdot 2\pi r L (P - U)$$

as before, or alternatively,

$$\frac{dQ}{dt} = b \cdot 2\pi r L (P - U).$$

Since $U = Q/\pi r^2 L$,

$$\frac{dU}{dt} = b \frac{2}{r} (P - U),$$

which on integration becomes

$$\frac{U}{P} = 1 - \exp \left[-\frac{2b}{r} t \right].$$

Putting $t = \pi r^2 L/v$,

$$\frac{U}{P} = 1 - \exp \left[-\frac{b \cdot 2\pi r L}{v} \right]. \quad (2)$$

Before proceeding to test equations (1) and (2) against the observations it is first necessary to define the units to be used. Time will be measured in minutes and length in millimetres; volumes are therefore measured in μl . Rates of urine flow have been quoted in $\text{m}\mu\text{l./min.}$ and must therefore be multiplied by 10^{-3} . The constants a and b have dimensions $\mu\text{l. mm.}^{-2} \text{ min.}^{-1}$. It is unnecessary to define units of concentration since concentrations appear as the ratio U/P . It is also necessary to define a standard tubule. Tubules differ in diameter in different regions; in the proximal region the external diameter is of the order of 100μ and the internal diameter of the order of 50μ in a normal state of distention. The theoretical

treatment considers the wall of the tubule as being of negligible thickness. As a bold approximation the standard tubule will be defined as having a length of 20 mm. and a radius of 0.04 mm.

In the theoretical treatment the assumption was made that over any cross-section of the tubule the concentration of the diffusing substance is uniform. But in fact the concentration will be greater near the walls because (i) the substance is diffusing inwards through the walls, and (ii) the speed of the stream is greatest at the axis. The case of diffusion into an infinite cylinder has been treated by Hill (1928), and his paper provides a graph from which it can be ascertained that 90% of the equilibrium value for the concentration within the cylinder is attained in a time given by

$$t = \frac{0.34r^2}{k},$$

where r is the radius of the cylinder and k is the coefficient of diffusion in $\text{cm.}^2/\text{min.}$

In what follows it will be seen that the worst case is that of an experiment with urea in which a rate of perfusion of $0.625 \mu\text{l./min.}$ was used. For urea in water the coefficient of diffusion is $0.97 \text{ cm.}^2/\text{day}$ (*Handbook of Physics and Chemistry*). Taking r as 0.004 cm. we find $t = 0.485 \text{ sec.}$ The volume of the standard tubule is $0.1 \mu\text{l.}$ so that under perfusion at $0.625 \mu\text{l./min.}$ the time taken to traverse the tubule is 9.6 sec. If the flow within the tubule is laminar then the speed on the axis is twice the average speed. Putting the worst case in the worst possible way we can say that the time taken to traverse the tubule cannot be less than 4.8 sec. This exceeds the time taken to reach 90% equilibrium by a factor of almost 10, and for this reason the assumption in question is considered to be justifiable.

(1) *Effect of altering the rate of secretion of natural urine.* The rate of secretion of natural urine may be altered by altering the concentration of potassium in the medium; but since the permeability of the tubule to other substances is likely to be altered by changes in the balance of monovalent and divalent cations, the method must be used with circumspection. Both increase and decrease of the normal rate can be achieved by this method and it follows that the substance to be studied should have a normal U/P ratio of 0.5 if the test is to be performed to best advantage. For this reason sucrose was chosen. Media were made up as indicated in Table 6, from which it can be seen that the total concentration of monovalent cations is approximately the same in all media.

Table 6

(Solution X: 1 part serum, 1 part 300 mM./l. labelled sucrose; Y: Ringer having potassium replaced with sodium; Z: Ringer having sodium replaced with potassium; R: Ringer.)

Medium	Sucrose (mM./l.)	Na (m.equiv./l.)	K (m.equiv./l.)	Na + K (m.equiv./l.)
(1) 1 pt. X, 1 pt. R	75	15	15	30
(2) 1 pt. X, 1 pt. Y	75	25	4.5	29.5
(3) 1 pt. X, 1 pt. Z	75	2.75	27	29.75

The results of experiments using these media are presented in Table 7. For each pair of observations the constant b has been calculated for the standard tubule and the average value of b is 3.4×10^{-4} . Using this value of b the curve of equation (1) is plotted in Fig. 2 with the observed values for comparison. The observations are not incompatible with this curve, but it is clear that in relation to the variation between one tubule and another the range of R achieved is insufficient to test the fit of the observations.

Table 7

Medium	U/P	R	b
(1)	0.47	2.0	3.54×10^{-4}
	0.47	1.6	2.84
	0.50	2.3	4.58
	0.47	1.9	4.28
(2)	0.49	0.65	1.23
	0.84	0.43	4.52
	0.82	0.48	4.37
(3)	0.35	2.7	2.91
	0.38	2.5	3.04
	0.35	2.4	2.58
			Av. 3.39×10^{-4}

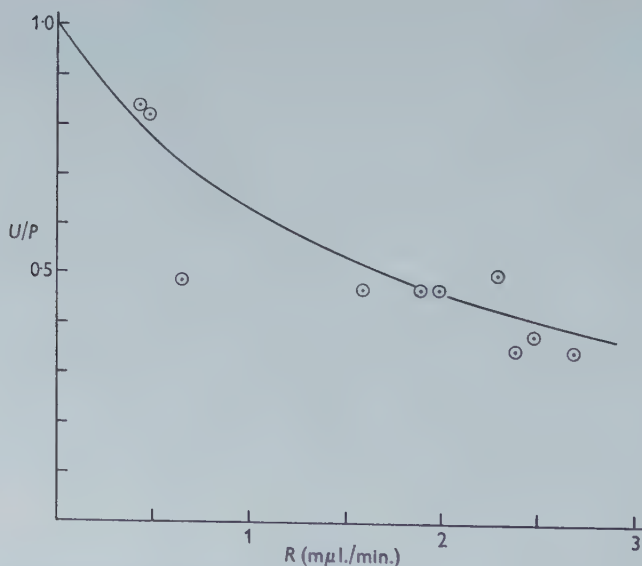


Fig. 2. The relation between the U/P ratio for sucrose and the rate of secretion of natural urine.

(2) *Effect of perfusing with artificial urine.* The injection of artificial urine into tubules at controlled rates of the order of $\text{m}\mu\text{l./min.}$ presents certain technical problems. The obvious alternatives are either: (i) to use a mechanically driven syringe, or (ii) to use a variable head of pressure in conjunction with an appropriate resistance. The problems of design and construction arising under (i) appeared

formidable and effort was therefore directed towards the second alternative. A pipette which is suitable for penetrating the tubule has a relatively low resistance and the pressures necessary to produce the requisite rates of flow are comparable with the pressures necessary to overcome the effects of surface tension in the system. An adequate resistance was achieved by the use of liquid paraffin in a fine capillary, the paraffin acting as a piston to drive the artificial urine through the pipette. The

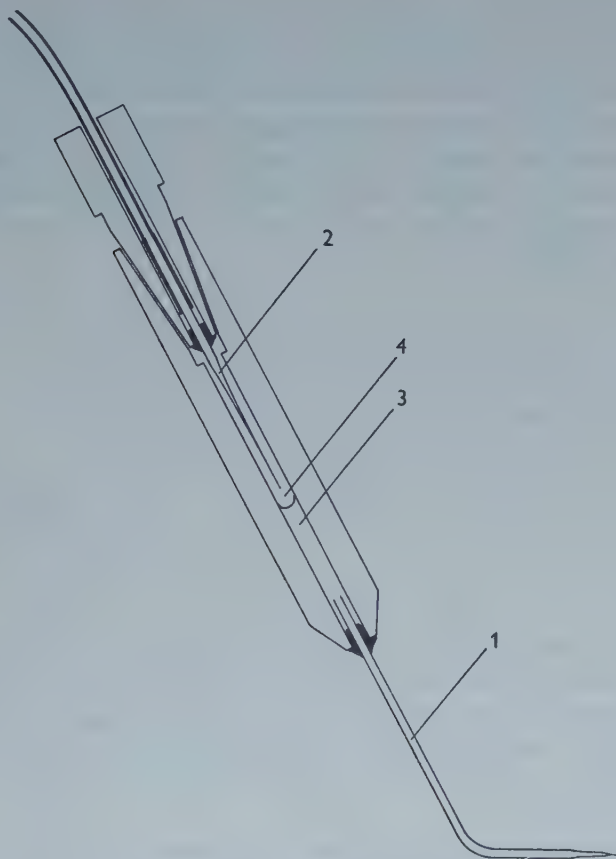


Fig. 3. Device for controlling rate of perfusion. 1, pipette for insertion into tubule; 2, fine capillary providing high resistance; 3, artificial urine; 4, liquid paraffin.

form of the injection apparatus is shown in Fig. 3. The silica pipette which penetrated the tubule was sealed into one end of a Perspex tube whose other end was provided with a tapered stopper. Into the stopper was sealed another silica tube, drawn out into a long fine point and connected to a nylon tube; these were filled with liquid paraffin. With precautions to exclude air bubbles the Perspex tube and pipette were partly filled with artificial urine; liquid paraffin was then added above the artificial urine and the stopper was placed in position. The other end of the nylon tube was connected to a screw-plunger device containing mercury and attached

to a mercury manometer. The Perspex tube was mounted on a micro-manipulator which is described elsewhere (Ramsay, 1953).

The artificial urine had the following nominal composition:

Sodium	5 m.equiv./l.	Phosphate	35 m.equiv./l.
Potassium	150	Chloride	150
Magnesium	30		

The tubule was set up in the usual way in a drop of medium containing the labelled substance, under liquid paraffin. The pipette was inserted at the distal end of the tubule and the perfusate was removed from the proximal end as it accumulated. After a suitable period of perfusion the volume of perfusate was estimated by measuring the diameter of the drop suspended in liquid paraffin, and the whole of the perfusate was taken for counting. The perfusion method has this advantage over the natural urine method that several observations at different rates of flow can be made on a single tubule.

Table 8. *Perfusion of tubule in medium containing labelled sucrose*

Tubule	Activity of medium, P , c.p.m./ $m\mu$ l.	Time of perfusion, t , min.	Volume of perfusate, V , $m\mu$ l.	Rate of perfusion, $R = V/t$	Count rate of perfusate, N , c.p.m.	Activity of perfusate, $U = N/V$	U/P	b	Equation
I	1.17	120	310	2.58	195	0.63	0.54	6.0×10^{-4}	(1)
		20	1190	59.5	53	0.044	0.038	4.6×10^{-4}	(2)
		240	270	1.12	240	0.89	0.76	7.1×10^{-4}	(1)
		30	2150	71.6	62	0.029	0.025	3.6×10^{-4}	(2)

The substance first studied by this method was sucrose and the results of one experiment are shown in Table 8. High and low rates of perfusion were compared, but the low rates are within the range of the rates of natural urine flow and it is probable that there was no perfusion under these conditions. Values for b were therefore calculated using equations (1) and (2) as appropriate, and are given in the table. As will be seen, the values are of the same order of magnitude, but the agreement is not good.

To realize the full potentialities of the perfusion method one should use a substance which penetrates rapidly so that useful values of the U/P ratio correspond to rates of perfusion which are many times greater than the normal rate of secretion. Of the substances used in this investigation the choice obviously falls upon urea. Table 9 and Fig. 4 give the results of experiments with this substance. The point on the ordinate was obtained from natural urine; for all the other points the rate of perfusion was more than $20 \times$ the normal rate of secretion. The curves of equation (2) are drawn for the average value of b in each experiment. For a total of four tubules tested the mean value (\pm s.d.) for b was found to be $4.5 \pm 2.5 \times 10^{-2}$.

It is of passing interest to compare the wall of the tubule with other natural membranes in respect of permeability to dissolved substances. Davson & Danielli (1952) give figures in terms of litres. μ^{-2} .sec. $^{-1}$ to which the present figures for b

may be converted by multiplying by 1.67×10^{-14} . For urea the value of $b = 4.5 \times 10^{-2}$ is used. For sucrose the value of $b = 6.7 \times 10^{-4}$ can be derived from the data in Table 4, using equation (1). The comparison is set out in Table 10, from which it is seen that the wall of the tubule is among the most permeable of natural membranes.

Table 9. *Perfusion of tubule in medium containing labelled urea*

(In all cases the concentration of urea in the medium was 53 mm./l.)

Tubule	Activity of medium, P, c.p.m./m μ l.	Time of perfusion, t, min.	Volume of perfusion, V, m μ l.	Rate of perfusion, R = V/t	Count of perfusate, N, c.p.m.	Activity of perfusate, U = N/V	U/P	b
1	0.312	30	1965	65	621	0.316	1.01	—
		15	2850	190	615	0.216	0.69	4.4×10^{-2}
		5	3125	625	303	0.097	0.31	4.6×10^{-2}
2	2.50	145	108	0.75	250	2.31	0.92	—
		5	470	94	609	1.30	0.52	1.4×10^{-2}
		5	1020	204	824	0.808	0.32	1.6×10^{-2}
		5	2470	494	1034	0.419	0.17	1.8×10^{-2}
3	0.297	10	2060	206	521	0.253	0.85	7.8×10^{-2}
4	0.297	30	1980	66	568	0.287	0.96	4.2×10^{-2}

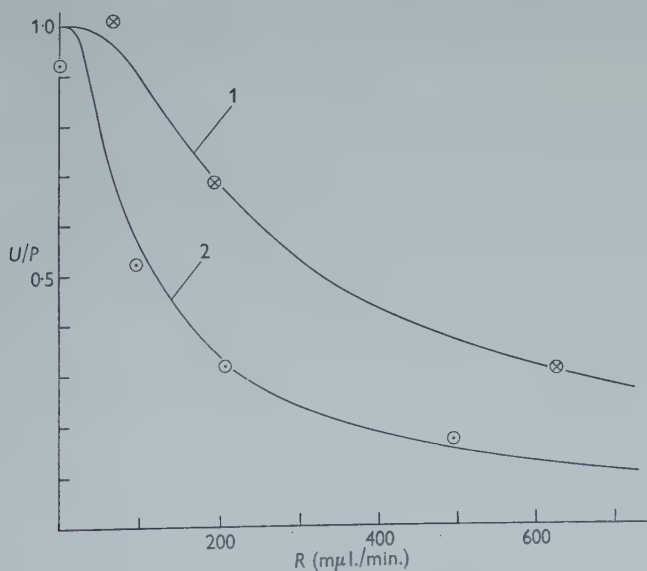


Fig. 4. The relation between the U/P ratio for urea and the rate of perfusion with artificial urine. Tubules 1 and 2 of Table 9.

Table 10. *Permeability of natural membranes of urea and sucrose*(Units: litres $\cdot \mu^{-2} \text{sec}^{-1}$. All figures should be multiplied by 10^{-10} .)

Membrane or organism	Urea	Sucrose
Ox erythrocyte	7.8	—
<i>Beggiatoa mirabilis</i>	1.58	0.14
<i>Chara ceratophylla</i>	0.11	0.0008
<i>Plagiothecium denticulatum</i>	0.0036	0.000008
<i>Dixippus</i> , Malpighian tubule	7.5	0.11

All figures, except for *Dixippus* Malpighian tubule, from Davson & Danielli (1952).

DISCUSSION

The evidence for the proposition that the organic substances used in this investigation enter the tubule by diffusion may be summarized as follows:

- (i) The U/P ratio is never greater than 1 (within the limits of experimental error).
- (ii) The U/P ratio characteristic of each substance is largely independent of P .
- (iii) The effects of rate of flow upon the U/P ratio are in accordance with expectation.
- (iv) There is no interference between different substances.

Other possible modes of excretion may be shown to be incompatible with this evidence. Let us suppose, for example, that:

- (a) The tubule transports in unit time a quantity, X , of substance, proportional to P . This gives a U/P ratio independent of P . The relation between U/P and R is

$$\frac{U}{P} = \frac{X}{R}.$$

If this were true then U/P should exceed 1 at low values of R , e.g. for sucrose (Table 7) at values of $R < 0.85$, which it obviously does not.

- (b) The tubule allows the substance to diffuse in very rapidly at some places and reabsorbs it at others; if U/P is to be independent of P it is necessary to suppose that a quantity, X' , proportional to P , is reabsorbed in unit time. On this theory an increase in the flow of natural urine would result in an increase in the U/P ratio, which is contrary to observation (Fig. 2).

It is clear that any alternative explanation which accounts for the observations is bound to be somewhat complicated, and in the absence of evidence to the contrary the truth of the proposition may be assumed.

Limiting rates of entry do not seem to have been reached under any of the experimental conditions tested, although these involved abnormally high concentrations and rates of flow. This, and also the absence of interference, suggest that the process is more likely to be one of simple diffusion rather than one of passive transport involving carrier molecules limited in number. On the other hand, the differences in rate of penetration as between one substance and another cannot be reconciled with any simple theory of membrane structure, e.g. the larger molecule of sucrose

(M.W. 342) penetrates more than three times as fast as the smaller molecule of glycine (M.W. 75). Lison (1942) was likewise unable to interpret the excretion of dyes by Malpighian tubules in terms of any well-known physico-chemical property.

In relation to the wider field of the physiology of excretion in insects the most significant observation is that to which reference was made in the Introduction, namely, that metabolically useful substances appear in the urine and can be reabsorbed in the rectum. Elementary text-books adhere to the traditional view in ascribing the excretory function in insects to the Malpighian tubules alone; a more enlightened view recognizes that the rectal glands can enter into the process of excretion by reabsorbing certain constituents of the urine, such as water and salts, and returning them to the haemolymph. The requirement for a brisk circulation through the tubule is obvious when one considers that solid particles, e.g. of uric acid, can only be removed from the tubules by being flushed out with a stream of water; in the rectum, an organ provided with muscles enabling solid contents to be evacuated, it is possible for the water to be almost completely removed. The requirement for a circulation of water and salts is easily understood, but it is not at once obvious that any advantage would accrue from committing metabolically useful substances to this circulation.

It may be permitted at this point to consider the principles of design that are applicable to excretory systems. Granted that a stream of water through the system is provided, either (i) we may secrete unwanted substances into the stream, or (ii) we may let all substances, wanted and unwanted, enter the stream and then reabsorb the wanted substances. A moment's consideration will show that if one function of our proposed excretory system is to remove foreign substances from the body then the second design is the better. We are not in a position to foresee what sorts of foreign substances we may one day wish to eliminate, and the first design requires that specific provision must be made to eliminate each unwanted substance; but on the basis of the second design unwanted substances are automatically eliminated simply by not providing specific mechanisms for their reabsorption.

The filtration-reabsorption process of the vertebrate glomerular nephron conforms to the ideal design. In recognizing the impossibility of filtration in the aglomerular nephron and Malpighian tubule we have perhaps overlooked the possibility of a diffusion-reabsorption process whereby soluble substances of small molecular weight enter the urine by diffusion rather than by being forced through a filter. The filtration-reabsorption process, which is available to vertebrates by virtue of their closed blood system and high arterial pressure, should be very much more efficient than the diffusion-reabsorption process to which insects are condemned by their open blood system and inadequate arterial pressure. In insects one would expect that selective advantage would lie with a tubule in which a rapid rate of urine formation was combined with a high permeability to dissolved substances, not necessarily excluding those that are metabolically useful.

Although the tubule can actively transport substances such as potassium ion and certain dyes, it is not altogether clear how far active transport of organic substances may be a part of the tubule's normal activity. More uric acid can be recovered from

the urine than from the same volume of haemolymph, but in view of the complicated solubility relations of uric acid and its salts one cannot say with certainty that uric acid is transported against an electrochemical gradient. The excretion of dyes has been intensively studied, notably by Lison (1942, and earlier papers), and there is no doubt that many of these, e.g. phenol red, are actively concentrated by factors which Lison estimates as of the order of 10,000. There are striking parallels between the Malpighian tubule and the vertebrate nephron in their behaviour towards dyes; in the kidney tubules of the flounder Puck, Wasserman & Fishman (1952) have measured concentration factors for phenol red as high as 4000. It is not easy to see why natural selection should have operated to provide this one class of substances with such an efficient mechanism of elimination or how this observation can be assimilated to any theory of the normal operation of the excretory system. It may be that the ability to concentrate dyes is an incidental property of some other feature of the excretory mechanism.

The importance which is currently attached to the purely thermodynamic distinction between active transport on the one hand and diffusion (simple or facilitated) on the other, and also to the kinetics of passage through membranes, tends perhaps to distract attention from other features of the process of secretion which are less amenable to interpretation in physico-chemical terms. Biophysicists have confined their attention to the passage of dissolved substances through cell membranes and have shown little interest in cells which liberate their secretions by rupture. Recent electron microscope studies of the Malpighian tubules of the grasshopper by Beams, Tahmisian & Devine (1955) show that the brush border is formed of an array of tubes $3-4\ \mu$ in length and less than $1\ \mu$ in diameter. Mitochondria are seen in various positions relative to the tubes, and the evidence suggests strongly that they are in process of migrating through the tubes, eventually to be set free into the lumen of the Malpighian tubule. Wigglesworth (1943) describes the excretion of biliverdin by the Malpighian tubules of *Rhodnius* in the form of small discrete masses which assume an elongate form as they pass through the brush border. These observations raise doubts as to the value of physico-chemical models for the interpretation of secretion at the cellular level in Malpighian tubules.

At the level of organ physiology it is now suggested that the role of the Malpighian tubules may be re-defined as follows. The tubule is not concerned in the regulation of the composition of the haemolymph. The tubule is not an organ which selectively removes only specific substances from the haemolymph; it is a means whereby all soluble substances of low molecular weight are indiscriminately removed from the haemolymph, to which they may be selectively returned by the rectal glands. This does not exclude the selective excretion of substances such as uric acid. The whole process of excretion in the insect involves the contributory processes of diffusion, reabsorption and secretion—in the vertebrate, of filtration, reabsorption and secretion. In the vertebrate filtration is exclusive to the glomerulus, reabsorption and secretion taking place in the tubule; in the insect reabsorption takes place mainly in the rectal glands, diffusion and secretion taking place in the tubule.

SUMMARY

1. Contrary to expectation, it is found that metabolically useful substances pass into the urine and are reabsorbed in the rectum.
2. The kinetics of penetration have been investigated for six amino acids, three sugars and urea.
3. The evidence indicates that these substances enter the urine by passive diffusion.
4. The role of the Malpighian tubules in the insect's excretory system is discussed in the light of these findings. It is suggested that the tubule is primarily a means whereby all soluble substances of low molecular weight are removed from the haemolymph and that in this respect it has analogies with the glomerulus as well as with the tubule of the vertebrate nephron.

I am very grateful to Dr K. E. Machin for criticizing the theoretical part of this paper and for valuable suggestions for improving the presentation of the results, most of which I was glad to accept.

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THE ADAPTABILITY OF MICE TO HIGH ENVIRONMENTAL TEMPERATURES

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(Received 7 May 1958)

The adult morphology of some mammals and birds has been shown to be determined by the temperature at which they are reared (Przibram, 1925; Sundstroem, 1930; Allee & Lutherman, 1940). In particular, mice raised at high environmental temperatures have much longer tails, larger feet, less fur and are usually smaller *when mature* than mice kept under temperate conditions (Sumner, 1909; Sundstroem, 1922; Ogle, 1934; Sakharov, 1949; Harrison, Morton & Weiner, unpublished). Harrison, Morton & Weiner have shown that the magnitudes of some of these heat responses, i.e. the differences between the environmental forms, are dependent upon the genotype of the animals, but apart from body weight, no exceptions to the above generalizations were found by them.

The aim of this investigation is to ascertain whether these morphological characters, and the physiological ones which are also known to be determined by environmental temperature, facilitate survival in the environment that produced them, i.e. are adaptive, or whether they have no effect on, or reduce, somatic fitness, i.e. are 'passive' (Harrison, 1958*a*). One method of determining the appropriateness of the high-temperature phenotype is to compare the heat tolerance of heat-reared animals with those reared at a lower temperature. Although there is no absolute distinction between the morphological and physiological components of the heat response, a particular structure, once formed, usually has a biological significance which is independent of the way it was formed; and whereas environmentally induced physiological changes are typically rapidly reversible, morphological changes, which are strictly a function of growth, are irreversible once the structure concerned has stopped growing and are usually only slowly reversible if still growing. To distinguish between morphological and physiological components in the heat response seems therefore justifiable, and they are to some extent experimentally separable. Some attempt is also made to separate the effects of single characters within the morphological response and to determine the way in which an effect is produced.

MATERIAL AND METHODS

C57BL \times RIII F_1 hybrid mice, bred at a dry-bulb temperature of 21° C. and an Assman wet-bulb temperature of 16° C. and weaned at 3 weeks of age, were reared either at the temperature at which they were bred (control-reared) or at 32° C. D.B.,

29° C. w.B. (heat-reared). A split-litter experimental design was employed, using body weight as the criterion for allocating animals into one or other of the environments, the two sexes being treated separately. Full details of this design are given in Harrison (1958*b*). The heat tolerance of the animals at an age of 8 weeks was determined by measuring survival time and death order at 41.7° C. D.B. ($\pm 0.3^\circ$ C.) 29.4° C. w.B. ($\pm 0.5^\circ$ C.). This temperature, when combined with a moderate air speed, is not too high to conceal small differences in fitness nor too low to make the experiment so protracted as to be unmanageable.

The exposures were performed in a well-insulated room, heated by the input of circulating hot air and steam, and controlled by electronic thermostats. Permanent records of the room temperatures throughout an exposure were made on a Honeywell Brown potentiometer. The animals, in shallow open-topped cages with holes bored in two of the sides, were placed at a standard height in a current of hot air maintained by a series of fans which reduced both horizontal and vertical temperature stratification to a minimum. The cages were arranged with their holed sides at right angles to the current. In this way it was possible to keep thermal conditions inside the cage identical with those outside. All the males in any one exposure were kept in a single cage and all the females in another, the two cages being placed side by side. The conditions were controlled as rigorously as possible because a great deal of evidence has been obtained that when an animal is living at the limit of its temperature regulatory capacity very small variations in temperature have profound effects.

The attempt to partition the causation of any difference in the survival time of control-reared and heat-reared animals into strictly morphological and physiological effects was undertaken by transferring animals from one environment to the other, 48 hr. before the heat-shock exposures. This procedure provided two further types of treatment 'transferred to control' and 'transferred to heat'. The choice of animals for transference was based on a method similar to that used at weaning. There is no reason to suppose that by 48 hr. 'transferred to heat' animals were as physiologically acclimatized to the conditions as those reared in the high-temperature room, nor that the 'transferred to control' animals had completely lost their high-temperature acclimatization, but by this time the rectal temperatures of the animals transferred to the heat were very similar to those of litter-mates reared in the heat. Although a longer period between transference and heat shock might have produced a closer similarity in the physiology of the transferred and indigenous animals, it would also have tended to effect a morphological similarity which was unwanted.

The effects of a tail on survival were investigated by amputating at 3 weeks of age the tail of a few C57 \times RIII hybrids, and comparing the performance of these animals with normal litter-mates in a heat-tolerance test, after both had been reared in the hot environment until 8 weeks old. The tails were removed with a sterilized scalpel at their junction with the body and the wound cauterized. Even immediately after recovery from the anaesthesia the animals show no concern about the wound and there can be little doubt that 5 weeks later any operative shock has been overcome.

Before a heat-shock exposure the animals were weighed. At precisely the same time the animals from the control and hot rooms were taken into the lethal temperature environment and animals of the same sex were immediately put in the same cage, so that all were subject to the same temperature fluctuations. The transference unavoidably involved the exposure of the animals from the hot environment to control conditions for 4-5 sec. The animals were provided with both food and water, but on only two occasions were individuals observed to drink. At death each animal was weighed, and in two of the exposures they were also weighed at 1, 5 and 7 hr. after the beginning of the tolerance test.

RESULTS

An analysis of survival time is complicated by the facts that (1) there is some evidence that the survival time of control-reared animals is bimodally distributed; (2) an analysis of variance, ignoring the effects of this distribution, reveals a significant difference in the survival time of at least the control-reared animals in different exposures; this is in part, but not entirely, due to the better control of the environment in some of the later exposures, following an unavoidable reconstruction of the lethal temperature room; (3) in some of the exposures there is also a significant difference in the survival times of identically treated animals in the two cages, but this cannot be interpreted as a sex difference in resistance since either of the sexes may be the longest lived; (4) some of the animals, particularly in the later exposures when the temperature fluctuations were smaller, survived for so long that the experiment had to be concluded before their death (e.g. > 15 hr.).

As a consequence, the actual survival times of heat-shocked animals in different exposures and in different cages cannot be combined in a comparison of the effects of differences of treatment. It is possible, however, to make within-exposure and within-sex comparisons of the order of death and to combine the frequencies with which a particular event occurs in different exposures and in different cages. Since it is also possible that there are litter differences, the comparison is made a within-litter one as well. The death order is established from the mean survival time of similarly treated animals, and the two sexes are treated as if in different litters. The probability of obtaining any particular frequency is determined by combining the appropriate terms of the binomial expansion

$$\frac{|n|}{|x| \cdot |y|} p^x \cdot p^y \cdot *$$

In those comparisons, where the frequency of one type of event is not significantly different from the frequency of the complementary type, rank numbers are given to the differences in survival time of the means compared, according to their magnitude

* When only one type of event occurs on a number of occasions, the total probability of this occurring by chance is the ultimate term of the expansion, i.e. p^n . When both types of event occur, the probability of the particular frequency is added to the probability of the less probable frequencies.

(Wilcoxon, 1945). The observed magnitude of the difference is a function of the severity of the exposure, so to offset the bias that could be introduced from this source the observed magnitude is scaled as a percentage of the mean survival time of the control-reared animals in the exposure. Admittedly this assumes that survival time is linearly related to the severity of an exposure; this may not be the case, but over a small range of conditions it is probably very nearly true. The effects of the four different treatments on survival are compared by this method in Table 1.

Table 1. *Comparison of the death order of differently treated C57 × RIII hybrid mice at 41.7° C. D.B., 29.4° C. W.B.*

(H=heat-reared, C=control-reared. Arrows indicate transference.)

Comparison		No.	No. in which A lives longer than B	Probability of no. diff.	Mean B % of A survival times
A	B				
H	H → C	9	5	≈ 1/2	≈ 108
H	C → H	9	9	1/512	< 76
H	C	11	11	1/2048	< 68
H → C	C → H	12	8	≈ 1/5*	< 86
H → C	C	9	9	1/512	< 78
C → H	C	10	9	1/93	76

* But < 1/20 by the ranking method explained in the text.

There is very strong evidence that the untransferred heat-reared animals survive in the high lethal temperature environment much better than either the untransferred control-reared ones or those that were transferred to the hot environment 48 hr. before the heat shock. There appears, however, to be no significant difference* in the survival times of heat-reared and 'transferred to control' (H → C) animals. On the other hand animals transferred to the heat (C → H) survive significantly longer than litter-mates kept in the control conditions until the heat-tolerance test. The one case in which the present experimental results are not clear cut is in the comparison of the death order of animals transferred to the control and those transferred to the heat. Since there is no significant difference in the survival of the heat-reared and the 'transferred to control' animals, and since the former live very significantly longer than animals transferred to the heat, one would have expected that the latter would not live as long as the 'transferred to control' group. Yet in four litters out of the twelve tested the mean survival time of the animals transferred to the heat was greater than that of those 'transferred to control'. However, if the ranking method of Wilcoxon is applied to the scaled differences in survival time, the 'transferred to control' animals are found to survive significantly longer than the 'transferred to heat' animals. (It is impossible to allot rank numbers to the differences in survival of heat-reared and 'transferred to control' animals since in many cases one or more animals did not die during the exposure. But even if

* Throughout 'significant' refers to < 5 % probability level.

rank numbers could have been allotted, whatever their distribution, no significant difference in the effects of these two treatments would have been found with the particular frequencies observed.)

The survival of the tailed and tailless litter-mates are compared in Table 2.

Table 2. *Within-litter comparison of death order of heat-reared tailless and tailed C₅₇ × RIII F₁ Hybrids*

Litter	No. of tailless animals	Mean body weight (g.)	No. of tailed animals	Mean body weight (g.)	No. of tailless animals dead before death of first tailed	Probability
1	2	18.0	2	18.9	2	1/6
2	1	19.1	1	22.1	1	1/2
3	1	16.4	1	17.3	1	1/2
4	1	17.0	1	18.0	1	1/2
5	1	22.5	1	21.8	1	1/2
6	2	14.4	3	15.3	2	1/10
Total	—	—	—	—	—	1/960

In every within-litter comparison the tailless animals die before the normal ones. Since the presence or absence of a tail is apparently the only distinguishing character, this experiment clearly demonstrates the survival value of a tail in withstanding high temperatures.

During a heat-tolerance test animals lose a lot of weight before death occurs. The absolute total loss is a function of initial size, so to facilitate a comparison of animals of different weight the loss per gramme of body weight has been calculated. The analysis of variance of the weight loss of control hybrids reveals that there are significant differences in the different exposures. It will be recalled that a significant difference was also found in the survival time of these animals in different exposures. There is a significant positive correlation between the mean survival times of males and the mean losses in weight per unit body weight in the different exposures ($r = +0.77$ (7 D.F.)); the correlation coefficient of the females is also positive ($r = +0.57$ (6 D.F.)) though not significant at the 5% level.

The losses in weight of similarly treated males and females are compared in Table 3. This has been done by calculating the mean loss in each exposure and then obtaining from these an over-all mean for the different exposures. Because of the differences in weight loss in the different exposures the means are balanced so that comparisons are based on results from the same exposures.

The comparisons reveal that there is no significant difference in the weight loss of similarly treated males and females when allowance is made for differences in original weight. The results for the two sexes have therefore been combined in the comparison of the effects of treatment, which shows that the weight loss of control-reared animals is not significantly different from that of heat-reared ones. Since the control-reared animals typically have a shorter survival time than heat-reared litter-mates, it follows that they lose weight more rapidly.

Table 3. Comparison of the mean loss in weight (g./g. of original body weight) of similarly treated male and female C57 × RIII hybrids and of differently treated C57 × RIII hybrids in heat-tolerance tests

Comparison						D.	S.E.	't'
Mean	No.	S.E.	Mean	No.	S.E.			
Control♂♂			Control♀♀			0.035	0.0255	N.S.
0.177	7	0.0168	0.212	7	0.0190			
Heat♂♂			Heat♀♀			0.002	0.0206	N.S.
0.175	8	0.0156	0.177	8	0.0134			
Control♂♀			Heat♂♀			0.011	0.0215	N.S.
0.187	9	0.0158	0.176	9	0.0145			

The rate of loss was studied in two exposures by weighing animals at 1, 5 and 7 hr. after they were put in the lethal temperature environment. Since there is no significant difference in the total loss of control-reared and heat-reared animals, the results have been expressed as the percentage lost, at each of these times, of the total loss at death, and the mean litter means are recorded in Table 4.

Table 4. Mean percentage loss of total loss in weight at 1, 5 and 7 hr. after the onset of two heat-tolerance tests

Exposure	Control-reared				Heat-reared			
	1 hr.	5 hr.	7 hr.	Survival time (min.)	1 hr.	5 hr.	7 hr.	Survival time (min.)
1	52.5	85.6	90.2	431	41.0	70.9	82.4	554
2	48.6	72.0	88.2	557	39.9	55.5	66.7	699

As one would expect from these figures there is a negative correlation between the amount of weight lost by an animal in the first hour of an exposure and its survival time. Using all the animals, irrespective of treatment, in these two exposures, the correlation coefficient in one of them is -0.41 , which on 33 degrees of freedom is significant at the 5% level, and in the other is -0.35 , which on 23 degrees of freedom is approaching significance at this level. Unfortunately, the correlations are not sufficiently good to allow one to use loss in weight in the first hour as a reliable measure of the heat tolerance of an animal, but it does indicate that the animals which lose weight most slowly have the greatest chance of a long survival.

DISCUSSION

The results demonstrate very clearly that the changes which occur when mice of the genotype C57 × RIII are reared at 32° C. D.B. 29° C. W.B. instead of at 21° C. D.B. 16° C. W.B., facilitate survival at a yet higher temperature. It may be concluded,

therefore, that the over-all environmentally determined response of the heat-reared animals is adaptive as tested in this way and that heat acclimatization is a very real phenomenon. Although the very nature of the test makes it impossible to state categorically that the forms produced by the two environments are the forms best fitted to these environments, the evidence suggests that this is probably the case.

The fact that the 'transferred to heat' animals survive significantly longer than their control-reared litter-mates confirms the reality of a physiological component in the acclimatization, since little or no morphological change occurred during the 48 hr. which these animals spent in the hot room. It is probable, indeed, that the bimodal distribution of the survival time of control-reared animals is due to the acquisition of physiological acclimatization during the heat-tolerance test itself. The nature of the physiological changes has not been investigated in the present work, but has been rigorously studied in other comparably treated mammals (Robinson, 1952; Findlay & Beakley, 1954). The 'transferred to heat' animals may not be as physiologically acclimatized to high temperatures as the heat-reared ones; nor can it be assumed that the 'transferred to control' animals have lost all their physiological acclimatization to the heat. However, the fact that not only the heat-reared but also the 'transferred to control' animals survive longer in a heat-tolerance test than the animals transferred to the heat is a strong indication that the morphological component in the phenotype is itself adaptive. Indeed, if the transferred animals have become as physiologically acclimatized to their new environments as the 'native' animals, it must be concluded that the morphological heat responses are more important in survival than the physiological ones. This of course does not mean that within the morphological category itself all the components are adaptive.

A few mice of other genotypes, both inbred and F_1 hybrid, have been tested in a similar way to the $C57 \times RIII$ hybrids. In each case control-reared animals survived less well than heat-reared ones. Too few animals were, however, transferred from one environment to the other to permit a systematic partition of the adaptability into physiological and morphological components, but in all genotypes save one the results were consistent with the above conclusions.

Where the responses to the environment are adaptive the lability of the phenotype will presumably have its own particular genetic basis, determining the extent and direction of the environmental modification of development, and if all the environmentally caused differences in phenotype of different genotypes in the same environment are adaptive, then these differences will represent the diverse capacities or requirements of the various genotypes for adaptation.

The most striking morphological difference that distinguishes control-reared and heat-reared animals is tail length, and since the morphological component of the heat-response has been shown to be adaptive and the possession of a tail has such a profound effect on heat tolerance, it seems likely that variations in tail length will change an animal's thermoregulatory capacity. (It is somewhat surprising that the body weights of the tailed and tailless animals are so similar, after allowance is made for the weight of the tail. This suggests that at least up to temperatures

of 32° C. other mechanisms can compensate for the absence of a tail when the rest of the environment is near optimal.) Whereas one might expect a tail of the appropriate length to become genetically fixed in some climates, in a fluctuating one the lability itself may have considerable value. Thus, for instance, although a long tail may be of adaptive value in the summer, it cannot easily become genetically fixed in the population if the fittest winter animal is a short-tailed one. The dependence of tail growth on temperature, however, is likely to provide successive generations with the tail length appropriate to the conditions prevailing when they begin breeding.

At an environmental temperature of 32° C., 4° or 5° C. below that of the body, the value of a long richly vascular tail functioning as a heat radiator is self-evident, but since there are no sweat glands in the mouse its value at an environmental temperature above that of the body is not so obvious, and it might in fact have been expected that under these conditions a long tail would increase heat gain rather than heat loss. It must be concluded that there is effective insensible perspiration, although no convenient way of demonstrating it has so far been devised. Such a conclusion is confirmed by the findings of Njaa, Utne & Braekkar (1957) who have discovered indirect evidence that there is a considerable passage of water across the tail of the rat in a dry environment, and it has been shown on innumerable occasions that insensible perspiration is responsible for much of the water-loss in man and his domestic animals (Du Bois, 1927; Kuno, 1934; Findlay, 1950).

The role of the other extremities in heat regulation has not been studied but one would expect them to function in a similar way to the tail. The effects of environmentally caused differences in body size on temperature adaptation are impossible to determine experimentally, since it is not only difficult to isolate weight differences from the physiological and other morphological changes that also occur, but, being a character which reflects vigour, one cannot distinguish between its own biological function and the multitude of other functions which determine it. Even, for instance, were it shown that genetically small animals were better adapted to the heat than genetically large ones, it would not necessarily mean that a reduced growth rate at high temperatures is an adaptive response. Indeed, there is indirect evidence (Harrison, Morton & Weiner, unpublished) that it is not, in which case genotype differences will not represent different capacities to adapt, but different abilities to buffer development against environmental effects.

Loss of water must be mainly responsible for the loss in weight during a heat-tolerance test. That there is a significant difference in the weight loss in different exposures suggests that animals may die when there is still water available for evaporation. The positive correlation between the severity of an exposure and the total water-loss indicates, in fact, that animals die when they can no longer lose water rapidly enough to maintain their body temperature. The final cause of death would appear then to be heat stroke, but this is a consequence of progressive dehydration.

Since the total water available for evaporation is the same in control-reared and heat-reared animals, the better survival of the latter is likely to be mainly due to

their slower loss of this water. They may lose water more slowly either because they have less metabolic heat to lose or because such water as is lost is used more efficiently. The physiological components of high-temperature acclimatization appear to function principally in the reduction of metabolic heat production, but the morphological adaptations can only influence heat loss. One might expect that the loss of water by insensible perspiration across the skin, particularly across the naked skin of the tail, feet and ears, is a more efficient way of using water for cooling than by panting and rubbing saliva in the fur. If this is so then the longer tail and feet, more richly vascular ears, and scantily covered body skin of the heat-reared animals makes their thermoregulatory capacity more efficient than that of the control-reared animals. Certainly their better survival in a heat-tolerance test favours such a conclusion.

SUMMARY

1. $C57 \times RIII F_1$ hybrid mice reared at 32°C. D.B. , 29°C. W.B. , from 3 to 8 weeks of age, survive longer at $41.7^\circ \text{C. D.B.}$, $29.4^\circ \text{C. W.B.}$, than litter-mates reared at 20°C. D.B. , 16°C. W.B.
2. The transference of animals from the hot environment to the cooler one 48 hr. before they are exposed to the lethal temperature has little or no effect on their heat tolerance; but transference in the opposite direction greatly increases survival time.
3. Mice whose tails have been amputated 5 weeks before they are exposed to the lethal temperature have a lower heat tolerance than normal animals.
4. The total loss in weight of an animal exposed to the lethal temperature is independent of the environmental temperature at which it has been reared; but heat-acclimatized animals lose weight less rapidly than control ones.
5. It is concluded that at least some of the changes, both physiological and morphological, which occur when mice are reared at high temperatures, are in their over-all effect adaptive.

I am very grateful to Dr J. S. Weiner for his help and advice and to Mr C. W. Graham for his invaluable technical assistance. I should also like to thank Dr D. S. Falconer for supplying the breeding stock of mice and Dr D. Michie for sending me an abridged translation of P. P. Sakharov's paper.

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FURTHER STUDIES ON IONIC REGULATION IN THE MUSCLE FIBRES OF *CARCINUS MAENAS*

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(Received 29 July 1958)

INTRODUCTION

When a euryhaline crab like *Carcinus maenas* is living in dilute sea water, the blood concentration is markedly reduced and, as a consequence, all the animal's cells must be able to adapt themselves to these new conditions. In attempting to analyse this process of cell adaptation it is necessary to know the normal processes by which the ionic composition of the cell is maintained and the extent to which these are modified under the conditions of reduced blood concentration.

Earlier papers (Shaw, 1955 *a, b*) described the normal ionic composition of the muscle fibres of *C. maenas* and the changes in composition which occurred when the crabs were adapted to diluted sea water. Under these conditions the reduction in blood concentration is reflected in alterations in the concentration of many of the muscle constituents. The muscle sodium and chloride concentrations are reduced in about the same proportion as their respective concentrations in the blood, but there is a marked retention of calcium, magnesium and potassium. In the case of calcium and magnesium there is reason to suppose that they are retained simply as a result of the almost complete impermeability of the fibre membrane, but this simple hypothesis is not likely to be an adequate explanation in the case of potassium. A more acceptable hypothesis is that the potassium ions are held in the fibre by the electrostatic charge of the non-chloride anion fraction to which the membrane is impermeable, and that these ions are selected in preference to sodium ions because of an active mechanism for the exclusion of the latter.

This paper describes investigations: (1) to determine the nature of the non-chloride anion fraction and the permeability of the muscle fibre membrane to this; (2) to measure the permeability of the fibre membrane to potassium and to explore the mechanisms for its retention; and (3) to throw some light on the mechanism of sodium exclusion.

MATERIAL AND METHODS

The supply and maintenance of the animals and the analytical methods were as described previously, except for additional estimations described below. In all analyses the muscle fibres were prepared free from extracellular fluid and the measurements are of intracellular concentrations.

Phosphate

Phosphate estimations were made on trichloroacetic acid extracts of the muscle by the colorimetric method of Fiske & Subbarow (1925), as detailed in Delory (1949). The various phosphate fractions were determined as follows:

(a) *Inorganic phosphate*. The crabs were chilled to 0° C., the muscles quickly removed, weighed and transferred to ice-cold 8% trichloroacetic acid and ground with sand. The extract was filtered in the cold and immediately neutralised. The phosphate content was measured directly, without precipitation of the inorganic phosphate, since arginine phosphate in the presence of ammonium molybdate is hydrolysed very slowly at room temperature (Baldwin & Yudkin, 1950).

(b) *Arginine phosphate*. A trichloroacetic acid extract of muscle at room temperature was diluted to 2% acid and the phosphate ester hydrolysed in a boiling water-bath for 3 min.—solution contained orthophosphate derived from inorganic phosphate and arginine phosphate and the arginine phosphate was determined by difference.

(c) *Adenosine triphosphate*. The remainder of the hydrolysed extract was made normal with respect to hydrochloric acid and further hydrolysed for 10 min. in a boiling water-bath. This hydrolysed the last two phosphate radicals on the adenosine triphosphate molecule, and these were added to the phosphate already present in the solution. Again the ATP content was calculated by difference.

(d) *Other phosphate esters*. Hydrolysis was continued for 24 hr. in the normal acid solution. The total phosphate in the acid extract was also measured by neutralizing a known volume of the extract with sodium carbonate, drying and incinerating. The ash was taken up in acid and phosphate content measured. The phosphate content was also determined on trichloroacetic acid extracts of dried muscle fibres.

Arginine

This was measured in the diluted trichloroacetic extract of dried muscle by the colorimetric method of Macpherson (1946) modified by the replacement of the α -naphthol by hydroxyquinoline (Janus, 1956).

Tracer studies

The penetration of phosphate into the muscle was studied by the injection into the blood of crab-Ringer solution containing ^{32}P orthophosphate. Sodium and potassium penetration was followed by using Ringer containing ^{24}Na and ^{42}K respectively. The radioactivity was measured by the usual method using an end-window counter for sodium and potassium measurements and a liquid counter for phosphate. Counting errors were not greater than $\pm 3\%$.

Potential difference measurements

These were made with the aid of two calomel half-cells—the penetrating electrode being of the Ling & Gerard (1949) type. The potentials were recorded on a modified Pye pH meter.

RESULTS

(a) *Phosphate*

The measurements on the phosphate content of normal muscle fibres is shown in Table 1. The values for the phosphate compounds were derived from the assumption that the first phosphate fraction represents inorganic phosphate in the intact fibre, that the second fraction contained the additional phosphate liberated from arginine phosphate and the third fraction contained an additional two phosphate radicals derived from adenosine triphosphate. The extra phosphate which appeared after prolonged hydrolysis was then accounted for by the third phosphate from the ATP molecule and the fact that the total acid soluble phosphate is almost identical with the phosphate yielded by this prolonged hydrolysis indicates that phosphate compounds, other than inorganic phosphate, arginine phosphate and ATP are either completely absent or only present in traces.

Table 1. *The concentrations of acid-soluble phosphate compounds*

Phosphate fractions	Concn. (mm./kg. fibre water)	S.D.	No. of measurements
1. Free phosphate in cold TCA extract	23	3	8
2. Total free phosphate after 3 min. boiling 2% TCA	82	5	14
3. Total free phosphate after 10 min. boiling N-HCl	100	5	14
4. Total free phosphate after 24 hr. in N-HCl	109	9	8
5. Total acid-soluble phosphate	108	8	11
Phosphate compounds			
1. Inorganic phosphate	23	—	—
2. Arginine phosphate	59	—	—
3. Adenosine triphosphate	9	—	—
Arginine	81	4	14
Potassium	146	12	48

The total arginine content of the fibres shows a very close correspondence to the sum of inorganic phosphate and phosphagen-phosphate. Since arginine phosphate is known to be a labile compound readily broken down during muscle activity (Meyerhof & Lohmann, 1928*a*), it is by no means impossible that the normal method of estimating inorganic phosphate by extraction in the cold leads in fact to phosphagen breakdown, and that in the intact fibre the phosphate is actually all combined with the arginine in the form of the phosphagen.

The phosphate and arginine analyses are in general agreement with other analyses which have been reported for crustacean muscle. Robertson's (1957) analyses for the muscles of *Nephrops norvegicus* give values for intracellular concentrations of phosphate compounds (calculated from his figures, using 11.5% as extracellular volume and assuming absence from the blood) of 21.6, 84 and 13.7 mm./kg. fibre water respectively for inorganic, arginine phosphates and ATP. The value for

arginine phosphate is somewhat higher than in *Carcinus*, but this may almost certainly be associated with the higher intracellular potassium concentration. The inorganic phosphate concentrations are similar, and these values are also close to those found by Eggleton & Eggleton (1928) for crab muscle, and the percentage of phosphagen phosphate to inorganic and phosphagen phosphate in *Carcinus* (72%) is not very different from the value of 66% found by Meyerhof & Lohmann (1928*a*) for the abdominal muscle of *Astacus fluviatilis*. The arginine content of the muscles of a number of American crabs has been measured by Arnold & Luck (1933). In three species the concentration varied from 771 to 855 mg. arginine per 100 g. of muscle. This would correspond to a concentration of about 70 mM./kg. of intracellular water and would thus be only slightly smaller than the concentration found in *Carcinus* muscle.

Table 2. *The electrostatic equivalence of the potassium and the acid-soluble phosphate compounds in the muscle*

Substance	Mean concn. in muscle (mM./kg. water)	Equivalent concn. at pH 7 m-equiv./kg. water)
Arginine phosphate	82	84.5
Adenosine triphosphate	9	33.4
Total negative charges	—	117.9
Potassium	146	146
Excess negative over positive charges	—	28.1

It is generally supposed that phosphate compounds in muscle make up a large proportion of the total anions, and in some vertebrate muscles like rat muscle (Conway, 1950) and frog muscle (Boyle & Conway 1941) a good agreement has been found between total cations and measured anions. Potassium ions and the organic phosphate compounds make the most important contributions to the cation and anion fraction respectively. There seems little doubt that the same is broadly true of crustacean muscle. In *Carcinus* muscle sodium is largely balanced by an equivalent quantity of chloride (Shaw, 1955*a*). The contribution of the phosphate compounds to the remaining anion fraction can be computed from their concentrations (Table 1) and from a knowledge of the pH of the fibre interior. The latter has been measured directly by means of an intracellular glass electrode by Caldwell (1954), and found to be about 6.9. The results are shown in Table 2, where the number of charges per g. molecule were calculated from the pK values of the various phosphate compounds given by Meyerhof & Lohmann (1928*b*) and by Conway (1950). The values for potassium concentration in the fibres is rather higher than found previously (Shaw, 1955*a*). Assuming that all the arginine is combined with phosphate as phosphagen, there is an approximate balance between the positive charge of the potassium ions and the negative charges of the phosphate compounds. The excess of positive charges of some 28 mM./kg. fibre water could easily be accounted for by the negative charge on the cell proteins, and it is probably not necessary to postulate the presence of other small anion molecules.

Robertson's (1957) analysis of whole muscle of *Nephrops* also show a satisfactory agreement between the potassium and phosphate fractions.

With the identification of the main anion fraction within the muscle fibre it is now possible to explore the behaviour of this fraction under circumstances where the blood concentration is greatly reduced. Measurements of the concentrations of the various phosphate fractions were made on muscle of crabs which were kept in dilute sea water at concentrations down to 40% normal sea water.

Fig. 1 shows the relation between the total acid soluble phosphate of the muscle and the blood sodium concentration. There is a marked fall in the phosphate concentration as the blood level falls, but at the lower concentrations the fall is less marked. This type of relationship is similar to that found for the behaviour of the intracellular potassium under similar circumstances (Shaw, 1955*b*). In the latter

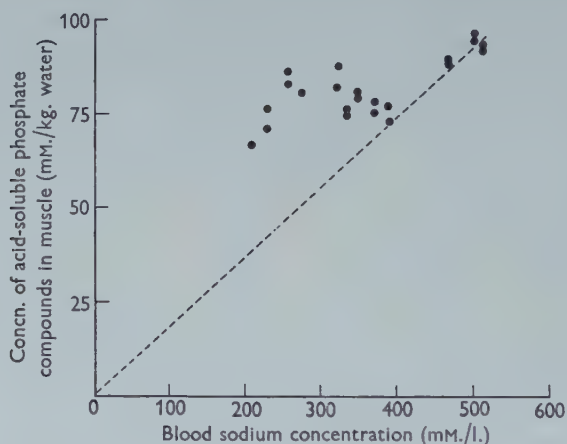


Fig. 1. The relation between the sodium concentration of the blood and the concentration of acid-soluble phosphate compounds in the muscle.

case the fall in concentration could be explained by the increase in water content of the muscle resulting from the dilution of the blood, and a similar explanation is adequate in the case of the muscle phosphate. When the acid-soluble phosphate is calculated on a dry-weight basis, and this is related to the blood sodium concentration (Fig. 2), it can be seen that there has been no significant loss of phosphate from the muscle to the diluted blood.

The simplest explanation of these findings is that the muscle fibre membrane is impermeable to the organic phosphate compounds and also to inorganic phosphate. The fact that the correlation between the arginine content of the muscle and the easily hydrolysed phosphate fraction is very high at all blood dilutions adds further evidence for the view that all the arginine is normally combined (Table 3). If permeability to any of these substances is assumed then active processes would have to be evoked to explain their retention. The concentrations of these compounds in the blood are very low, and their electrochemical potentials are in each case much

higher inside the fibre than outside. Such active processes would also have to be capable of regulation under conditions of blood dilution.

A formal demonstration of the impermeability of the fibre membrane to all of these substances has yet to be made, but tracer studies on the behaviour of inorganic phosphate lend further support to this hypothesis. Sodium orthophosphate labelled with ^{32}P was made up in crab-Ringer solution and injected into a number of crabs and in such a concentration as to only increase the normal blood phosphate level by about 0.2 mM./l. The crabs were kept out of water, but in a damp environment to prevent loss of radioactivity by diffusion into the surrounding sea water, for periods up to 12 days. At intervals during this period a crab was removed and an analysis made of a trichloroacetic acid extract of the muscle for phosphate and total radioactivity. This was compared with the phosphate content and radioactivity of a trichloroacetic acid extract of the blood.

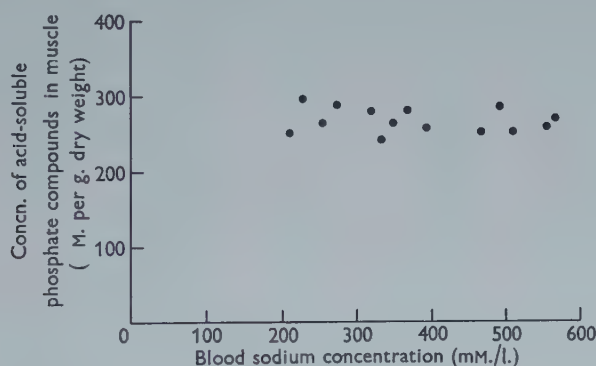


Fig. 2. The relation between the sodium concentration of the blood and the amount of acid-soluble phosphate compounds per g. dry weight of the muscle.

Table 3. *The muscle concentrations of arginine and the total inorganic phosphate after hydrolysis in 2% trichloroacetic acid*

Muscle no.	Arginine concn. (mM./kg. water)	Inorganic phosphate after TCA hydrolysis (mM./kg. water)
1	87	85
2	83	86
3	80	82
4	78	82
5	78	81
6	76	74
7	67	67
8	69	68
9	61	65
10	66	68

The ratio of the radioactivity in a given volume of muscle fibre water to the radioactivity in the same volume of blood is shown in Fig. 3 plotted against the time after the injection of the phosphate solution. After a few hours this ratio reaches about 10, then levels off and shows no further increase for the remainder of the 12 days.

Since the blood concentration is in all cases less than 0.5 mM./l. phosphate the ratio represents less than a 5% exchange with the total acid-soluble phosphate. If instead of the total phosphate, the 'inorganic' phosphate alone is considered then the ratio would indicate some 20% exchange with the cell inorganic phosphate. From studies on vertebrate muscle, however, there is every reason to believe that the phosphate radicals of the various compounds (with the exception of the third phosphate of ATP) are readily exchangeable with each other (see, for example, Hevesy, 1947) and the separation into the various fractions would be misleading. Furthermore, the rapid uptake of the radioactive phosphate within a few hours and the subsequent constancy does not suggest a permeability phenomenon but probably some kind of absorption.

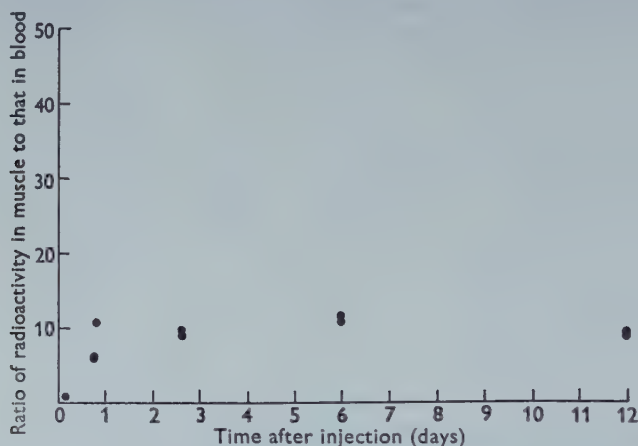


Fig. 3. The penetration of ^{32}P , as inorganic orthophosphate, into the muscle.

The behaviour of *Carcinus* muscle with respect to radioactive phosphate resembles in many respects that of frog skeletal muscle (Causey & Harris, 1951). In this case the radioactivity was shown, by autoradiography, to be located at the surface of the fibres. Generally speaking, no convincing evidence has been brought forward to show the permeability of any skeletal muscle to phosphate (Harris, 1956, p. 184).

(b) Potassium

The possibility that potassium was held in the muscle fibre because of the impermeability of the membrane to this ion was soon ruled out by a study of the potassium exchange between the blood and muscle, using ^{42}K as a tracer. In the first series of experiments crabs were kept in artificial sea water (made according to Pantin, 1946, p 64), but containing some ^{42}K introduced as an isotonic solution of the chloride. The crabs remained in this solution for 48 hr., which allowed ample time for full equilibration of the tracer between the sea water and the blood. At the end of this time muscles were removed and weighed. The total radioactivity and the potassium content of the muscle were measured and these were compared with the activity and potassium concentration of the blood on an equal volume basis. The

percentage of the muscle potassium which had exchanged was calculated from $P = 100A_iK_o/A_oK_i$, where A_i and A_o are the activities per kg. water of the muscle and the blood respectively, and K_o and K_i are the concentrations of potassium in the blood and muscle. The results of these measurements are shown in Table 4. It is clear that the whole of the muscle potassium is exchangeable with the potassium of the blood, and this contrasts in a striking manner with the behaviour of the phosphate compounds. The exchangeability of the potassium is in general accord with the results which have been obtained for vertebrate muscle. Although in the latter case low percentage exchanges have been recorded the exchange is generally complete when experimental conditions are adequate (see, for example, Creese, 1954).

Table 4. *The exchangeability of the muscle potassium*

Crab series no.	Muscle	Muscle potassium concn. (mm./kg. water)	Percentage of of potassium exchanged
1	L. extensor	134	101
	L. flexor	145	105
2	L. extensor	123	127
	L. flexor	124	123
3	R. extensor	126	97
	R. flexor	117	102
4	L. extensor	128	98
	L. flexor	122	100
5	L. extensor	126	90
	L. flexor	139	90
6	L. extensor	133	99
	L. flexor	121	99
			103*

* Mean exchange

The rate at which potassium ions penetrate into the muscle was also studied. In these experiments a number of crabs were each injected with about 1 μ c. of ^{42}KCl (this raised the blood potassium concentration by about 2mm/l.) and they were then kept out of water but in a damp environment for periods of up to 6 hr. At known times after the injection blood and muscle samples were taken from individual crabs and the percentage of the muscle potassium which had exchanged was calculated as before. These results are shown in Fig. 4. If the blood and muscle potassium concentrations are constant and there is a constant proportion of radioactive to normal ions in the blood, then the rate of penetration should follow a simple relation, $P = 100(1 - e^{-kt})$, where P is the percentage of the potassium exchanged, t , the time and k , the exchange constant. The measurements of the blood activity showed that after the initial mixing of the injected material (which took about 3 min.) the level remained fairly constant during the experimental period, so that the relation could be used to give, at least, an approximate indication of the rate of exchange. The experimental data (Fig. 4) is represented approximately by such a curve with the exchange constant = 0.4 hr.⁻¹.

It is interesting to find that a similar value has been found by Keynes & Lewis (1951) for the rate of exchange of potassium in the isolated nerve of *Carcinus*—they

find values of 0.49 hr.^{-1} for the whole nerve and 0.37 hr.^{-1} for the isolated 30μ axon. The diameter of even the largest nerve axons is, however, much less than that of the muscle fibres, so that although the internal potassium concentration of the nerve is higher, the potassium flux through the axon membrane must be appreciably smaller than through the muscle fibre membrane. Keynes & Lewis calculated that the axon membrane flux was in the order of $22 \times 10^{-12} \text{ M./cm.}^2/\text{sec.}$ —a similar calculation for the muscle fibre, using an exchange constant of 0.4 hr.^{-1} and a fibre diameter of 300μ allows a rough estimate of the flux as $130 \times 10^{-12} \text{ M./cm.}^2/\text{sec.}$ It may be of some significance that this flux is of a different order of magnitude from that which has been found in amphibian muscle fibres. Thus, for example, in the frog toe muscles and sartorius muscle the membrane fluxes are only 4.5 and $12 \times 10^{-12} \text{ M.cm.}^2/\text{sec.}$ respectively (Keynes, 1954). The large difference between the

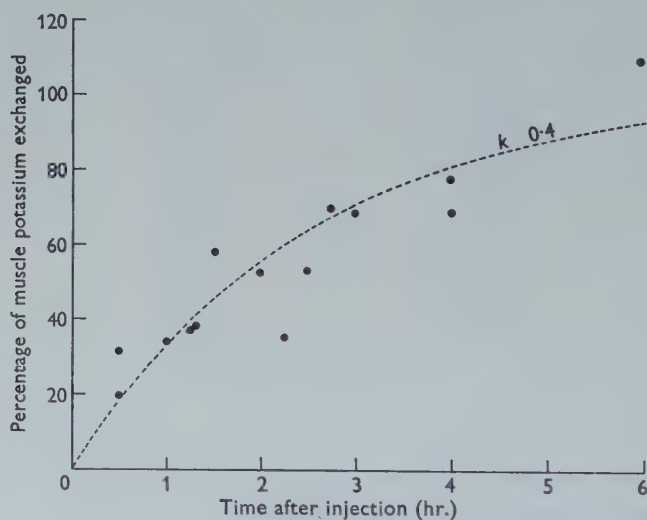


Fig. 4. The penetration of ^{42}K into the muscle.

permeability of the muscle fibre membrane to potassium in *Carcinus* and in the frog is confirmed by measurements of the transverse resistance of the membrane by an electrical method (Fatt & Katz, 1953). Their results show that *Carcinus* muscle has a low membrane resistance—a mean of 165 ohm.cm.^2 —compared with the higher values which have been found for frog muscle (Fatt & Katz, 1951; Castillo & Machne, 1953) which range from 1500 to 4300 ohm.cm.^2 .

The demonstration of the relatively high permeability of *Carcinus* muscle fibre membrane to potassium ions means that an explanation of the retention of this ion in the muscle must be sought among mechanisms which promote a dynamic steady state between potassium ions in the blood and the fibre. The simplest condition is where the electrochemical potential of the potassium ions is the same on either side of the fibre membrane and it was suggested previously that this is the case (Shaw, 1955*a*). This point has been re-investigated in view of the fact that (*a*) the muscle

potassium concentration was higher in the present series of crabs, and (b) the previously recorded membrane potentials were rather lower than had been recorded in the same muscles by Fatt & Katz (1953). In the present experiments membrane potentials were measured by the technique of Fatt & Katz, using a crab-Ringer solution for bathing the isolated muscle preparation. This technique gave better visibility than previously and had the advantage that any solutes leaking out from damaged fibres could be washed away by the large volume of solution. Damaged fibres were more easily seen and potential difference measurements were made only on those fibres which appeared completely undamaged. In this way many of the previously recorded low potentials were eliminated and this resulted in an increase in the mean recorded membrane potential. Values of this potential recorded from several muscles are shown in Table 5 and the mean value of 70 mV. shows a close agreement with that found by Fatt & Katz.

Table 5. *The effect of reduced blood potassium concentration on the muscle potassium concentration and on the membrane potential*

(Concentrations in mM./kg. water).

Blood K concn. (K_o)	Muscle K concn. (K_i)	Muscle Na concn.	Ratio K_i/K_o	Membrane potential (mV.)	'Equilibrium' potential (mV.)
(a) Muscles of crabs from normal sea water					
12.0	164	—	13.6	73	—
12.8	146	—	11.4	72	—
12.6	142	—	11.2	70	—
12.3	146	—	11.8	71	—
11.7	144	—	12.3	67	—
(b) Muscles of crabs from potassium-reduced sea water					
10.0	143	—	14.3	78	74
10.3	150	—	14.5	67	74
9.5	150	—	15.8	73	77
9.5	137	—	14.4	76	74
9.2	137	—	15.0	70	75
8.8	138	—	15.6	77	76
9.0	138	—	15.3	75	76
7.6	127	—	16.7	78	78
7.6	135	—	17.8	82	79
7.8	126	—	16.2	—	—
6.7	137	—	20.5	85	83
4.9	100	61	20.4	—	—
6.4	128	72	20.0	76	82
4.0	110	74	27.5	75	89
5.5	115	65	21.0	78	83
5.2	130	78	25.0	79	88
5.8	111	65	19.2	82	82

The nature of the distribution of the potassium is best tested by comparing the membrane potential with the 'equilibrium potential' derived from the expression, $E = 58 \log C_i f_i / C_o f_o$ where C_o and C_i are the blood and muscle potassium concentrations respectively, and f_o and f_i , their activity coefficients. The calculation can only be made if the ratio, f_i/f_o is assumed to be unity. The mean value for the equilibrium potential, calculated in this way, comes out to be 63 mV., compared with the membrane potential of 70 mV. As it is unlikely that the discrepancy between

the two can be due to experimental error, if the values are accepted as they stand it would be necessary to postulate the presence of some active process transporting potassium ions out of the fibre.

There are, however, at least two reasons why the derivation of this equilibrium potential may be misleading. In the first place, the assumption that the ratio of the activity coefficients is unity is probably not valid. The ionic strength, upon which the coefficients depend, will not be the same in the blood and the muscle. In the latter, only about one-third of the total osmotic pressure is made up of ions (Shaw 1955*a*). In the second place, it is quite possible that the muscle ions are not uniformly distributed throughout the muscle (this is discussed below). If potassium is restricted to the inner parts of the fibre, then the measurement of its internal concentration, within that region, would be too low. Both these factors would have the effect of increasing the calculated equilibrium potential and they would be of the right order to raise it up to the measured membrane potential. It is difficult to decide to what extent these corrections should be applied, but in view of them it is clear that further evidence would be required to establish the existence of a mechanism concerned with the active transport of potassium. In any event, the active transport of potassium ions would represent only a small fraction of the total potassium flux, and until more critical measurements can be made it is simplest to accept as a first approximation that the potassium ions are distributed according to the electrochemical gradient.

However, it must be clearly stated that the acceptance of the passive distribution of the potassium ions as a working hypothesis does not imply that these ions cross the fibre membrane by simple diffusion process, nor does it characterize the membrane potential as a simple diffusion potential.

The question of the retention of potassium in the muscle under conditions of blood dilution can now be considered. It was shown previously (Shaw, 1955*b*) that, in crabs from dilute sea water, the muscle potassium concentration was reduced in proportion to the intake of water into the fibres. There was generally no loss of potassium from the muscle except, perhaps, at the more extreme dilutions. The behaviour of the muscle potassium in general resembles closely that of the acid-soluble phosphate compounds and, in fact, as is shown in Fig. 5, there is always a very close correlation between the muscle potassium concentration and the total concentration of the acid-soluble phosphates, both in the normal muscle and in the muscles from diluted blood.

Under conditions of extreme blood dilution the ratio of muscle potassium to blood potassium is greater than normal (Shaw, 1955*b*). It was suggested that if the membrane potential was constant, some active component was concerned with the retention of potassium. Again, however, the detection of the presence of an active mechanism turns critically on the measurements of the membrane potential and on the calculation of the equilibrium potential. More favourable conditions for the analysis of this problem can be found by reducing the blood potassium concentration, but keeping the total blood concentration constant. In this way dilution of the muscle potassium by the intake of water is prevented and large initial muscle-

to-blood potassium ratios can be achieved. These conditions were realized by keeping crabs in artificial sea water with a reduced potassium concentration, for periods of a week or longer. At the end of this period, in each crab the membrane potentials were measured in the muscles from one chela and the muscles of the other chela were analysed for potassium content and, in some cases, sodium content. The equilibrium potential for the potassium ion (E') was calculated on the assumption that this ion is passively distributed in the normal muscle, and hence, from the expression $E' = 58 \log C_i/C_o + E_c$, where E_c is the small potential (7 mV.) which must be added to the first term to make E' equal to the measured membrane potential in the normal muscle. The results of these analyses are shown in Table 5.

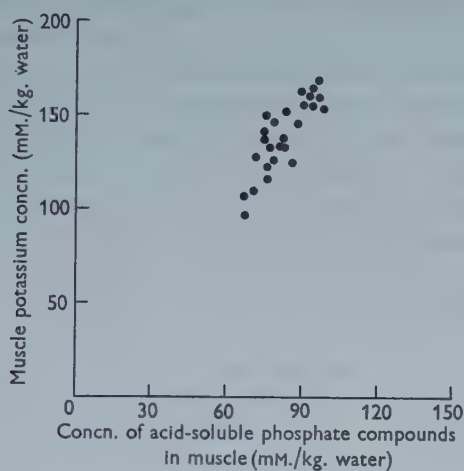


Fig. 5. The relation between the concentration of potassium and of the acid-soluble phosphate compounds in the muscle.

From the results of the effects of reduced blood potassium concentrations several important conclusions can be drawn. The muscle potassium concentration is not wholly maintained; it shows a significant fall which is most marked (a drop of 25%) when the blood potassium concentration is lowest. Despite this, however, the ratio K_i/K_o , is increased from the normal value of about 12 to a value of 20 or more. The loss of potassium is accompanied by the entry of sodium. In most cases the increase in sodium concentration (from the normal of 54 mM./kg. fibre water) is not as great as the fall in potassium concentration and some anions are presumably also lost. Finally, the membrane potential of the muscles is significantly increased. It is obvious that if the distribution of potassium remains passive, then the new membrane potential must be equal to the equilibrium potential (E'). In many cases this is so; in some, particularly at the lower blood potassium concentrations, the equilibrium potential is somewhat higher than the measured membrane potential. The possibility therefore that there is some small active component in the potassium influx cannot therefore be ruled out. However, it is certain that if any active mechanism for the uptake of potassium is present, it can account for only a small fraction of the total

potassium influx and is quite inadequate to maintain the normal muscle potassium concentration under these conditions. This applies even more forcibly in the case of the blood dilution, where the K_1/K_2 ratios do not generally exceed about 1.7.

It is abundantly clear that the really important factor which is responsible for the retention of potassium in the muscle is the rigid exclusion of sodium ions. The efficiency of this mechanism, whatever form it may take, is maintained during blood dilution and only begins to show signs of break-down at very low blood potassium concentrations.

(c) Sodium

The exchangeability of the muscle sodium with sodium of the blood was measured by injecting a number of crabs, each with a small amount of crab-Ringer solution containing about 20 μ c. of ^{24}Na . The animals were subsequently kept out of water but in a damp environment, and at known times after injection muscles were removed, separated into individual fibres and washed to avoid contamination with blood. The percentage of sodium exchanged was calculated from measurements of sodium concentration and radioactivity per unit volume of water, as described for the similar experiments with potassium. The results are shown in Table 6. The exchange between the blood and muscle sodium is very rapid and complete. The difference between the mean value for the exchange ($97 \pm 2.7\%$) and a complete exchange is not significant.

Table 6. *The exchangeability of the muscle sodium*

Crab series no	Muscle	Time after injection (min)	Muscle sodium concentration (mm. Na^+ water)	Percentage sodium exchanged
1	L. extensor	0.5	46	100
	R. extensor	0.5	30	97
2	L. extensor	1.0	38	100
	R. extensor	1.0	44	76
3	L. extensor	1.5	41	96
	R. extensor	1.5	22	97
4	L. extensor	2.0	22	100
	R. extensor	2.0	11	96
5	L. extensor	2.2	46	100
	R. extensor	2.2	30	100
				96

* Mean exchange

Even an approximate measurement of the rate of penetration of ^{24}Na into the muscle was impossible by this method. Only 3 min. after the injection as much as 70% of the sodium had exchanged and the exchange was complete in 10 min. Since it took several minutes for the injected sodium to become evenly distributed throughout the circulating blood, the bulk of the exchange occurred at a time when the blood activity was far from constant. For this reason it is not possible to measure the exchange constant but, on the other hand, it is quite clear that the penetration rate is extremely rapid.

A widely accepted view holds that low muscle sodium concentrations are maintained by the active extrusion of sodium ions from the muscle fibre. For a steady state, the rate of sodium extrusion (sodium efflux) must equal the rate of sodium penetration (sodium influx). If, in *Carcinus* muscle, the rate of penetration of ^{24}Na is a measure of the normal sodium influx then a simple calculation shows that this theory is untenable—the energy requirement for the extrusion of sodium at the same rate and against the electrochemical gradient would be far too great. Other explanations must be looked for to account for the rapid exchange.

The explanation which immediately springs to mind is that an exchange diffusion, such as has been suggested by Ussing (1947), occurs between labelled and non-labelled ions. This type of interaction may be regarded as an artifact of the tracer technique and will lead to estimates of ion fluxes which are too large. Exchange diffusion will not affect net movements of the ions in question.

To test for the presence of exchange diffusion, net movements of the muscle sodium were induced by perfusing chelae with a Ringer solution containing 50% or more of isotonic dextrose or choline chloride. As shown in Table 7, a rapid loss of sodium resulted and after 10 min. perfusion some 40–50% of the muscle sodium was lost. On these grounds it seems unlikely that exchange diffusion can account for more than a small part of the rapid sodium exchange.

Table 7. *The loss of sodium from muscles perfused with sodium-reduced Ringer*

Perfusing fluid	Na concn. of perfusing fluid (mm./l.)	Perfusion time (min.)	Na concn. of muscle after perfusion (mm./kg. water)	Na concn. of normal muscle (mm./kg. water)
Dextrose-Ringer	246	10	31	53
	246	15	32	53
	246	30	40	57
	27	15	21	57
	27	10	23	53
	27	10	18	83
	27	10	18	83
Choline-Ringer	230	4	29	60
	230	7	35	63

The loss of sodium in the perfused muscle is quite compatible with the behaviour of the muscle sodium under conditions of blood dilution (Shaw, 1955 *b*), where the fall in muscle sodium concentration is in proportion to the decrease in blood sodium concentration.

How, then, can these facts be reconciled with the maintenance of a low muscle sodium concentration? No certain answer can be given—it is possible, however, to erect a hypothesis which is in accord with the facts, such as they are. One may suggest that the muscle sodium is not uniformly distributed throughout the fibre, in association with all the other muscle ions, but is situated in a special region or compartment which is outside the boundary across which the membrane potential is developed. This special region would be in close contact with the blood and the sodium within would be at approximately the same concentration as in the blood.

The relatively small volume of the region would account for the fact that measurement of sodium concentration on the basis of the fibre as a whole leads to a value much less than that of the blood.

The idea that a muscle fibre may consist of more than one region is, of course, by no means new. Several authors have reached the same conclusion with regard to the behaviour of sodium ions in amphibian muscle. Conway (1954) first suggested that the muscle sodium was divisible into several fractions and, later (Conway & Carey, 1955) that a rapidly exchanging fraction was located in the sarcolemma. Since then, Simon, Shaw, Bennet & Muller (1957), Edwards & Harris (1957) and Harris (1957) have all brought forward additional evidence which suggests that the muscle is divisible into at least two compartments, one of which contains the bulk of the muscle sodium. Harris locates the rapidly exchanging sodium in an outer annulus surrounding the interior of the fibre.

The two concentric compartments suggested in Harris's hypothesis would seem applicable to *Carcinus* muscle, and, here, the sodium would be confined to the outer compartment and rigidly excluded from the inner. If the sodium concentration in the outer compartment was the same as in the blood, in a muscle fibre of $300\ \mu$ in diameter, the outer annulus would be about $7\ \mu$ in thickness. This is considerably thicker than the sarcolemma and there is no obvious correlation with any morphological location. The mechanism of exclusion of sodium from the inner compartment still remains unknown, but this, presumably is an energy-requiring process which works less efficiently at low blood potassium concentrations.

DISCUSSION

In attempting to describe the processes of ionic regulation in the normal muscle fibre of *Carcinus*, one must be careful to avoid accepting too simple a picture. It is noteworthy that in amphibian striated muscle, despite over 10 years of intensive research by many workers, the position still requires clarification.

In *Carcinus*, from normal sea water, each muscle fibre contains large amounts of relatively indiffusible organic phosphate compounds. These are negatively charged and the charge is approximately balanced by the high concentrations of potassium ions. The retention of potassium ions appears to be largely electrostatic—if an active process is concerned in potassium transport, it can account for only a very small part of the total potassium flux. The retention is a consequence of the permeability of the membrane to potassium and the efficient exclusion of sodium from the fibre. Undoubtedly it is the mechanism by which sodium exclusion is achieved which is the most important single process of ionic regulation in the muscle fibre. This is true wherever the muscle sodium is located—if, however, the freely-exchangeable sodium is indeed present in the outer layers of the fibre, then the process of sodium exclusion must lead to a practically complete absence of sodium from the fibre interior.

The distribution of the muscle sodium requires further investigation, as does the behaviour of the muscle chloride ions.

Despite the fact that a complete and certain picture of the processes of ionic regulation in the normal muscle fibre cannot yet be given, it is possible to attempt to explain the effect of dilution of the blood upon the composition of the muscle.

Dilution of the blood results in the intake of water into the muscle, and this brings about a fall in the concentration of both the muscle phosphates and the potassium—there is no attempt to maintain the normal concentrations of these substances. The behaviour of the phosphate compounds appears to be purely passive and the same is probably true of the potassium ions. There may be some minor active component in the potassium influx but this is quite insufficient to maintain the normal potassium concentration in the fibre.

The fact that during moderate blood dilution the blood potassium concentration falls roughly in proportion to the fall in muscle potassium concentration insures that the ratio of muscle to blood potassium, and also the membrane potential, remain substantially unaltered. This parallel fall in muscle and blood potassium concentrations may be purely fortuitous—but it is clear that the regulation of blood potassium concentration, as well as the concentrations of sodium and chloride, in animals from dilute sea water is of great importance for the maintenance of cell potassium levels.

Sodium ions remain excluded from the muscle and there is no evidence of interference with this mechanism under moderate dilutions of the blood. Changes in the muscle sodium concentration can best be explained as due to the rapid loss of sodium from the outer layers of the fibres.

Under conditions of extreme blood dilution, such as is found in animals living in less than 30% sea water where survival is poor, additional changes in the muscle composition are observed. The very low blood potassium concentration leads to higher muscle-to-blood potassium ratios and a consequent additional loss of potassium from the fibres. The low potassium also appears to interfere with the normal sodium exclusion mechanism and this, in turn, may lead to the penetration of some sodium into the fibre interior.

In relation to the general problem of the adaptation of the muscle to reduced blood concentrations, these studies show that, as far as the muscle ions are concerned, the cell behaves rather passively. The presence of special regulatory processes concerned with the maintenance of the normal ionic composition of the cell has not been revealed.

SUMMARY

1. The muscle fibres of *Carcinus maenas* contain 91 mM./kg. fibre water of acid soluble phosphate compounds, of which arginine phosphate is the most abundant. These compounds behave passively under conditions of blood dilution and the phosphate radicals do not exchange freely with inorganic phosphate in the blood.
2. Muscle potassium exchanges freely and completely with the blood potassium. The exchange constant is about 0.4 hr.^{-1} . In the normal fibre potassium ions are approximately in equilibrium with those in the blood. Potassium is lost from the muscle when the blood potassium concentration is reduced, but both the muscle-to-blood potassium ratio and the membrane potential are increased. The behaviour

of potassium appears to be largely passive but there may be some small active component in its retention.

3. Muscle sodium exchanges very rapidly and completely with blood sodium. The exchange is complete in 10 min. Sodium is also lost rapidly from the muscle to Ringer solutions with a reduced sodium concentration. It is suggested that the bulk of the muscle sodium is situated in a region distinct from the true fibre interior and that sodium ions are rigidly excluded from the latter.

4. These results are discussed in relation to the adaptation of the muscle fibre to reduced blood concentrations.

This work was assisted by a grant from the Royal Society.

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OSMOREGULATION IN THE MUSCLE FIBRES OF *CARCINUS MAENAS*

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(Received 29 July 1958)

INTRODUCTION

In considering the general problem of the penetration of marine animals into brackish water and ultimately into fresh water, it is clear that even in animals, like *Carcinus maenas*, which are able to maintain the concentration of their body fluid significantly above that of their environment in dilute sea water, all the cells of the organism must be able to adapt themselves to considerable changes in the composition of their surrounding fluid. In *Carcinus*, for example, living in 40% sea water the blood concentration drops to about 60% of normal. The process of cellular adaptation to blood dilution is quite unknown. It is, however, possible to visualize several ways by which this might come about. On the one hand, the cells may take up large amounts of water during blood dilution, and adaptation may take the form of increased tolerance to cell hydration. On the other hand, the cells may maintain their normal composition by the maintenance of the cellular osmotic pressure—a process which would involve the removal of water entering by osmosis. Although it has been generally assumed that cells are always isosmotic with their surrounding fluid, processes for the removal of water are known in the Protozoa and probably exist in the cells of fresh water sponges and coelenterates. It has also been suggested that certain mammalian organs contain cells which are hyperosmotic to the body fluids, although this still requires confirmation (Opie, 1949; Robinson, 1950). Finally, the process of adaptation may involve a reduction in the cell osmotic pressure by the removal of some of the osmotically active constituents sufficiently rapidly to prevent the excessive intake of water.

From previous work on the ionic composition of the muscle under different conditions of blood composition (Shaw, 1955*a, b*, 1958) some of these possibilities can be eliminated. In the first place, the change in water content of the muscle fibres in crabs from dilute sea water is not sufficient to account for the behaviour of the fibres on the basis that they are simple osmometers. In the second place, there is no evidence to show that the concentrations of the muscle ions are regulated to any extent; they behave passively with respect to water movements.

Two possibilities remain—either that the osmotic pressure of the fibre is maintained above that of the blood or that the osmotic activity of the fibre is reduced by the removal of some part of the non-ionic fraction of the muscle. This latter fraction is quite extensive—the muscle ions account for only about one-third of the total

osmotic pressure (Shaw, 1955*a*). In the decapod Crustacea this fraction has long been associated with the presence of large quantities of nitrogenous substances. Thus Duchâteau & Florkin (1955) find from 4427 to 5708 mg. amino acids per 100 g. muscle in marine *Eriocheir*. Kermack, Lees & Wood (1955) find a similar range of free amino acids together with trimethylamine oxide and betaine in the lobster, and Robertson (1957) finds 500 mg./kg. muscle amino-N and 100 mg./kg. trimethylamine oxide in *Nephrops* muscle. In *Carcinus* muscle, Lewis (1952) identified glycine, alanine and taurine by qualitative chromatography.

The possibility of regulation of the concentration of these substances is apparent from the fact that in freshwater decapods like *Astacus* (Camien, Sarlet, Duchâteau & Florkin, 1951) and *Eriocheir*, adapted for long periods to fresh water (Duchâteau & Florkin, 1955) the concentrations of some of the free amino acids may be much lower than in the marine forms. Lack of information on the water content of the muscle of the latter makes it impossible to know the cause of the reduction in concentration.

This paper describes investigations directed towards (i) the elucidation of the mechanisms by which adaptation to reduced blood concentration is achieved in the muscle, and (ii) the distinction between the maintenance of osmotic pressure and the reduction of osmotic activity.

MATERIAL AND METHODS

Experimental details with regard to the crabs followed those in previous papers (Shaw 1955*a, b*). The muscles used were, as before, the extensor and flexor of the carpopodite of the chela. Muscles were prepared as groups of single fibres, and in such a way that all analyses refer to the composition of the muscle fibres—not to the whole muscle, including trapped blood.

Freezing-point depression. Measurements on blood and on single muscle fibres were made by means of the technique of Ramsay (1949) as modified by Ramsay & Brown (1955). For making measurements on individual fibres, crabs were cooled in a cold room at -3°C ., muscles were removed and single fibres dissected out in liquid paraffin at this temperature. The fibres were introduced into glass capillaries and then frozen at -80°C . and stored at this temperature until required for measurement. In this way it was hoped that autolytic changes occurring in the fibre after removal were reduced to a minimum.

Amino acid nitrogen. This was measured on tungstic acid and trichloroacetic acid extracts of fresh or dried muscle by means of Folin's method (Folin, 1922, as modified by Danielson, 1933). Examination of standard solutions of expected nitrogenous compounds showed that the method gave quantitative results for glycine, alanine, taurine and the α -amino group of arginine, but was negative for trimethylamine oxide and betaine.

Trimethylamine oxide. This was reduced to ammonia by acid stannic chloride (Kermack *et al.* 1955). The ammonia was removed by diffusion and estimated by a micro-conductimetric method (Shaw & Staddon, 1958).

Non-protein nitrogen. By micro-Kjeldahl digestion and estimation of ammonia as above.

RESULTS

(a) *Total osmotic pressure*

Measurements of the freezing-point depression of single muscle fibres of crabs from normal and diluted sea water are shown in Fig. 1. It is clear that the measurements are consistent with the view that the total osmotic pressure of the muscle

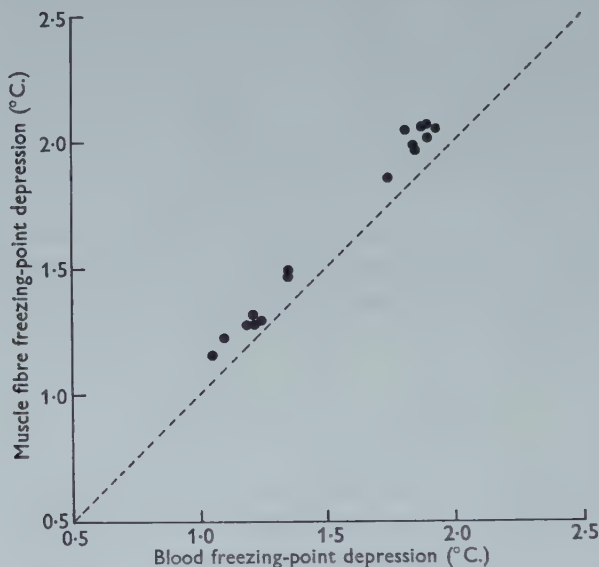


Fig. 1. The relation between the freezing-point depression of the blood and of the muscle fibres.

fibre falls in proportion to that of the blood when this is diluted. It is, however, noteworthy that in every case the fibre appears to be slightly hyperosmotic to the blood—generally about 7–8% higher. This was invariably found, both in the normal crabs and in those from dilute sea water. There was no evidence that this difference increased as the blood was diluted. It is important to consider whether this difference can be regarded as normal or whether it is an artifact of the technique. Although the temperature during preparation and measurement was kept below zero, it has been shown that autolysis may take place at 0° C., even in mammalian tissues (Conway, Geoghagen & McCormack, 1955). If autolysis did occur the most likely substances to be involved would be the labile phosphate esters—arginine phosphate and adenosine triphosphate. The concentrations of these substances in the muscle (Shaw, 1958) is just about sufficient to give the observed increase in concentration if they were broken down. The following observations add weight to this suggestion. If the fibres are separated and kept in liquid paraffin at room temperature for some hours before measurement, there is no increase in the apparent freezing-point depression. Further, if the fibres are dried (this is known

to bring about the breakdown of phosphate; Shaw, 1958) and then extracted with distilled water the water extract again (when corrected to same water content) shows the same degree of hyperosmoticity.

These considerations make it probable that the hyperosmoticity arises by autolysis after the removal of the fibres and that, *in vivo*, the muscle fibres are in osmotic equilibrium with the blood. In any event, the intracellular osmotic pressure always changes in proportion to a change in the blood concentration. There is no evidence for the maintenance of the fibre osmotic pressure in diluted blood.

THE COMPOSITION OF THE MUSCLE FIBRE

The inorganic ions of the muscle, together with the organic phosphate anions, only account for a relatively small proportion of the total osmotic pressure of the fibre (Shaw, 1955*a*, 1958). The remainder of the osmotic activity is due almost entirely to the presence of organic nitrogenous compounds. Some of these have been identified qualitatively as the amino acids, glycine and alanine, and the related taurine (Lewis, 1952). A quantitative analysis of the main nitrogenous compounds is shown in Table 1. The largest part of this fraction is accounted for by the

Table 1. *The concentrations of non-protein nitrogen-containing substances in the muscle fibres*

Substance	mean concn., mM./kg. fibre water	S.D. \pm mM/kg.	No. of measurements
α -Amino-N	516	47	15
Trimethylamine oxide-N	90	20	10
Arginine	82	—	—
α -Amino-N			
Arginine total N	328	—	—
Adenosine tri-phosphate total N	45	—	—
Non-arginine α -Amino-N (by difference)	434	—	—
Total non-protein N by addition	897	—	—
Total non-protein N by Kjeldahl	990	36	6
Unknown N compounds (by difference)	93	—	—

compounds containing amino nitrogen. These will include the above-mentioned substances, together with arginine, either occurring in the free state or liberated from its combination with phosphate. Trimethylamine oxide is also an important constituent, as was found also in lobster muscle (Kermack *et al.* 1955) and in *Nephrops* (Robertson, 1957). When all measured nitrogen is added up, together with

the contribution from adenosine triphosphate, the total falls somewhat short of the measured total non-protein nitrogen, estimated by the Kjeldahl method. The deficit amounts to 94 mM./kg. fibre water. This nitrogen may well be present largely in the form of betaine. This substance was found in concentrations of this order in the lobster muscle (Kermack *et al.* 1955), and, in view of the general agreement in the composition of the other known nitrogenous substances, it is a reasonable assumption this substance may also be present in *Carcinus* muscle.

Table 2. *The osmotically active components of the muscle fibre*

Substance	Concn., mM. or mg. ions/kg. fibre water
α -Amino-N compounds (excluding arginine)	434
Trimethylamine oxide	90
Unknown N-compounds (possibly betaine)	93
Potassium	146
Arginine- and inorganic phosphate	82
Adenosine triphosphate	9
Calcium	5
Magnesium	17
Sodium	54
Chloride	53
Total	983
Blood osmolar concn. (from freezing-point (m-osm./kg.))	1000

Making use of these analyses of the organic nitrogen compounds, together with the analyses of inorganic ions and phosphate compounds previously described (Shaw 1955, 1958), it is possible to see to what extent the concentrations of these substances can account for the total osmotic activity of the fibre. These concentrations are all listed in Table 2, and the sum of these (in mM./kg. water or mg. ions/kg. water, whichever is appropriate) can be compared with the osmotic activity of the blood at the same time, as measured by the freezing-point depression. The correspondence between the two is extremely good and the difference can scarcely be significant. However, in summing the concentrations of the muscle constituents in terms of osmotic activity, the assumption has been made that in every case the osmotic coefficient can be taken as unity. This is unlikely to be the case for the free ions—but even taking this into consideration the discrepancy between the muscle and blood is not likely to be greater than about 50mM./kg. water. It seems certain, therefore, that, together with the muscle ions, the organic nitrogenous compounds make up a very large part, if not the whole, of the total muscle osmotic activity.

MUSCLE COMPOSITION OF CRABS FROM DILUTE SEA WATER

With the identification of a very large fraction of the osmotically active components of the muscle it is now possible to study the regulation of the total osmotic activity of the muscle fibre in terms of the variations of the concentrations of the individual

constituents. The behaviour of the various muscle ions under conditions of blood dilution have already been described (Shaw, 1955*b*, 1958). We can now look at the composition of the muscle in crabs from dilute sea water with respect to changes in the concentrations of the nitrogenous fraction. Analyses of these compounds in muscles from crabs living in 40% sea water is shown in Table 3. The measurements of the concentrations of arginine and adenosine triphosphate are taken from Shaw (1958). It is evident that, by comparison with the similar analyses for the muscle of crabs from normal sea water (Table 1), every nitrogen-containing substance shows a marked and significant fall in concentration; and that this is especially true of the amino-N compounds, the concentration of which is approximately halved.

Table 3. *The concentrations of the nitrogenous compounds in the muscles of crabs from 40% sea water*

Substance	Concn., mM./kg. fibre water	S.D.	No. of measurements
Total non-protein N α -Amino N	611	44	6
α -Amino N	255	47	13
Trimethylamine oxide	58	10	6
Arginine	64	—	—
Adenosine triphosphate	8	—	—
Total arginine N	256	—	—
ATP-N	40	—	—
α -Amino N (excluding arginine)	191	—	—
Unknown N compounds (by difference)	66	—	—

In a similar study of the behaviour of the muscle ions during blood dilution (Shaw, 1955*b*, 1958), it was found that in dilutions of sea water at least down to 40% the changes in the concentration of the major ions (potassium and organic phosphates) could be explained solely on the grounds of the dilution of the muscle contents by the osmotic intake of water, consequent upon the dilution of the blood.

It is now necessary to see to what extent the changes in the other muscle constituents can be explained on the same grounds. To do this the concentration of these substances in the muscles of the 40% sea-water crabs were compared with the concentrations which would be expected on the assumption that water intake was the only factor determining their distribution. Water intake was calculated from the mean difference in water content of the muscles of normal crabs as compared with the muscles of crabs from 40% sea water. The results of these calculations are shown in Table 4. It is clear that in all cases the concentrations are below those expected from the change in water content and this is particularly marked in the case of the amino-N compounds. Here the drop in concentration is some 160mM./kg. greater than can be accounted for by water intake. This must mean that either these substances are removed from the fibre during blood dilution or they are combined

with other muscle constituents and no longer extractable with trichloroacetic acid. Another possible explanation that these substances were combined to form simple soluble peptides was found to be untenable. In the normal muscle, hydrolysis of the acid extract with N-HCl showed the absence of simple acid-soluble peptides. In the muscles from the 40% sea-water crabs there was likewise no increase in α -amino-N on hydrolysis.

Table 4. *The effect of the increased water content on the concentrations of the nitrogenous substances in the muscles of crabs from 40% sea water*

(Concentrations in mM./Kg. fibre water)

Substance	Mean concn. in muscles of crabs from 100% sea water	Mean concn. in muscles of crabs from 40% sea water	Concns. for 40% crabs calculated from change in water content	Loss not accounted for by change in water content
α -Amino-N compounds (excluding arginine)	436	191	355	164
Trimethylamine oxide	90	58	73	15
Unidentified N compounds	93	66	76	10
Water content (%)	74.0	77.8	—	—

The significance of the fall in α -amino-N concentration now becomes apparent. The measurements of the freezing-point depression of the fibre (Fig. 1) show that in 40% sea water (blood mean $\Delta = 1.2^\circ \text{C.}$) the osmotic activity of the fibre must fall from 1000 m-osm./kg. water to 650 m-osm./kg. water. The change in water content accounts for a fall of 200 m-osm. The difference is made up quantitatively by the loss of free α -amino-N compounds.

There is clearly a process operating for the reduction of osmotic activity within the fibre by the removal of free nitrogen-containing substances, chiefly amino acids and taurine. It is interesting to establish if this process is reversible, inasmuch as normal muscle concentrations are regained on the return of the crabs to normal sea water.

The demonstration that the concentration of the nitrogenous substances in the muscles of crabs which have been taken down into dilute sea water and then returned to normal sea water is unaltered, invites the criticism that in such animals the concentrations of these substances were, in fact, never changed, except by water movements. To meet this objection, measurements were made on individual crabs. as follows. A crab was adapted to 40% sea water, one chela was removed and its muscles analysed. The wound was allowed to heal and then the crab was returned to full-strength sea water. Finally the other chela was taken and the muscle analyses compared. The results of these measurements are shown in Table 5. The results are fully consistent with concept of the process as a reversible one. In all cases additional free α -amino-N compounds are made available, and the amount added (mean 173 mM./kg.) is comparable with the amount that was lost in the previous experiments (Table 4).

Table 5. The concentrations of substances containing α -amino nitrogen in muscles of crabs from 40% sea water and also in muscles after transference of the crabs to full-strength sea water

Concn. in muscle of crab from 40% sea water (mm./kg. water)	Concn. in muscle after transfer to 100% sea water (mm./kg. water)	Water content of muscle from 40% sea water (%)	Water content of muscle from 100% sea water (%)	Concn. expected from change in water content	Amount added (mm./kg. water)
271	508	76.9	72	348	160
286	537	76.5	73.7	332	205
266	533	80.3	74.2	377	156

DISCUSSION

It has often been suggested that free amino acids and other similar compounds play an important part in the osmotic activity of the cells of marine invertebrates. The analyses presented here and also the analysis of whole muscle of *Nephrops* by Robertson (1957) show that this is certainly the case. In *Carcinus* they account for over 60% of the total osmotic activity. Further it is now clear that in *Carcinus* these substances do not play an entirely passive role in the maintenance of cellular osmotic pressure but are actively concerned in the regulation of the osmotic activity. In discussing the adaptation of the muscle cells to diluted blood, from the point of view of the behaviour of the muscle ions (Shaw, 1955*b*) it was suggested that an important part of cell adaptation was the restriction of water uptake. It is now clear that this is not done by the maintenance of the intracellular osmotic pressure. The process involves the reduction of the internal osmotic pressure by the removal of osmotically-active components from the muscle. The process of reduction of internal osmotic activity achieves the same result, in terms of cellular hydration, as the maintenance of a high blood concentration.

Thus in *Carcinus*, as far as the muscle fibre is concerned, changes in the state of water content of the cell are restricted by two complementary processes—firstly, by the maintenance of the blood osmotic pressure above that of the environment, when this is diluted; and secondly, by a reduction of the osmotic activity within the cell itself. Thus, for example, in 40% sea water, the total effect of these two processes is to present to the cell conditions equivalent to a reduction in the concentration of the sea water to only 80% of the normal value. There is no reason to believe that the internal regulatory process is any less important than that operating on the blood. Indeed, in many brackish water animals with limited or no powers of osmoregulation (for example, *Arenicola*) the process of cell adaptation must be the dominant one and the development of such powers must play an important part in the penetration of marine animals into brackish waters.

The study of the process of cell adaptation shifts the emphasis from the importance of the maintenance of osmotic pressure, *per se*, to that of the water content of the

cell in relation to the osmotically inactive cellular components. Our present knowledge of the fine structure of cells, with their complicated array of thin membranes and vesicles, makes it apparent that any change in the water content could easily lead to disruption and distortion of these structures.

The question of the mechanism by which the reduction of the osmotic pressure exerted by the acid-soluble nitrogen compounds is effected in *Carcinus* muscle is an interesting one. It does not follow that these substances are removed from the fibre, it is only necessary that their osmotic activity be reduced. The fact that the process is reversible and that the blood concentrations of these substances are always low (less than 5 mM/l.) makes it a little difficult to see where the substances would be stored when they are temporarily removed from the fibre. The possibility that these substances are rendered osmotically inactive by their combination with large molecules of the cell, such as proteins, must be seriously considered and this would be worthy of further investigation.

As far as *Carcinus* is concerned one cannot consider the possession within the muscle of large amounts of nitrogenous substances which act as an osmotic reserve as a special adaptation for life in brackish water. Analyses of muscles of other, purely marine, decapod Crustacea, such as the lobster and *Nephrops*, show that these substances are present in these animals in much the same order of concentration. *Carcinus* has simply exploited an already existing situation.

The reason for the occurrence of the large concentrations of these nitrogenous compounds in the first place in the muscles of marine animals is more obscure. It is noteworthy that the ionic composition of the muscles of the animals in sea water is not very different in the concentrations of the main ionic constituents from that found in many terrestrial and freshwater animals. It is conceivable that there is an optimum concentration for ions in an efficiently operating striated muscle fibre, and it is possible that this is achieved in the marine forms by the addition of the organic nitrogen compounds.

Finally, it must be remembered that dilution of the blood affects all cells of the organism, not only the muscle fibres, and it does not follow that all cells behave in the same manner. Cells of the hepatopancreas of the lobster also contain large amounts of free amino acids (Kermack *et al.* 1955), but on the other hand the nerve fibres of *Carcinus* show a very different composition pattern from that of the muscle (Lewis, 1952). For this cell type, although free amino acids are present they are characterized by the large amounts of acidic acids, aspartic and glutamic in contrast to the muscle. These acids are balanced electrostatically by a much higher concentration of potassium. In this cell, adaptation to dilute blood must involve either a much greater change in water content than in the muscle or a substantial reduction in the intracellular potassium concentration. It seems reasonable to suppose that different cells may possess different powers of adaptability, and it may be that the death of the whole organism in very dilute sea water may be the result of the failure of one particular cell type to adapt itself to the new conditions.

SUMMARY

1. Measurements have been made of the freezing-point depression of single muscle fibres of *Carcinus maenas* and of the concentrations of the non-protein nitrogenous components of the muscle.

2. When the blood is diluted the osmotic activity of the muscle fibres always falls in proportion. The fibres are probably always in osmotic equilibrium with the blood.

3. The osmotic activity of the fibres can be accounted for in terms of the concentrations of the muscle ions together with nitrogen-containing compounds, such as free amino acids, taurine and trimethylamine oxide. These organic substances account for over 60% of the total osmotic pressure.

4. In crabs from dilute sea water the concentrations of the nitrogenous compounds are reduced below the level expected from the increase in water content of the muscle. It is suggested that the muscle fibre can prevent excessive water intake by the removal of nitrogenous substances, thus reducing the internal osmotic activity. The process is reversible.

5. The importance of the mechanism in relation to the adaptability of the cell to reduced blood concentration is discussed.

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OBSERVATIONS ON THE TEMPERATURE REGULATION AND FOOD CONSUMPTION OF HONEYBEES (*APIS MELLIFERA*)

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(Received 6 August 1958)

INTRODUCTION

The ability of a honeybee colony to regulate its temperature enables it to survive conditions to which individual bees succumb. The thermal preference of young bees varies from 35 to 37.5° C. and for older bees from 31.5 to 36.5° C., and depends to some extent on the temperature to which they have been previously conditioned (Heran, 1952).

In summer the temperature of the brood-nest of a colony is maintained at about 34–35° C. and fluctuates very little (e.g. Gates, 1914; Himmer, 1932). The bees normally prevent it from rising above this level by some or all of the following methods: the evaporation of droplets of water which they spread over the comb; the evaporation of water by manipulation of drops in their mouthparts; the creation of currents of air by fanning their wings; a proportion of them leaving the hive (e.g. Himmer, 1932; Lindauer, 1954).

Bees in the hive form a cluster which, as Gates (1914) noted, contracts and expands with decrease and increase in environmental temperature. The temperature in the centre of a broodless cluster in winter usually lies within the range 20–30° C. and is more often nearer to 30° C. than to 20° C. (e.g. Gates, 1914; Wilson & Milum, 1927; Corkins, 1930).

In the present experiments bees have been kept in groups of from 10 to 200 bees, at different environmental temperatures, and the temperatures maintained by the groups, and the amount of sugar syrup and water they consumed, were periodically measured. Records were also made of the approximate percentage of the bees of each group which were clustering together, and of the number which had died. All experiments were carried out during the winter months.

METHOD

The bees were kept in well-ventilated Perspex cages (6 × 5 × 9 cm. high). A thermometer, a glass gravity feeder containing sugar syrup (2 parts sugar : 1 part water by weight), and another containing water, were inserted through the roof of each cage at such angles that the ends of the feeders and the bulb of the thermometer nearly met at a point 1.5 cm. below the centre of the roof. Six wax-coated nails driven into the underside of the roof, so as to form a circle round the ends of the

feeders and thermometer, provided additional footholds for bees clustering there. A graduated scale attached to each feeder enabled the consumption of syrup and water to be measured.

Six cages containing 0, 10, 25, 50, 100 and 200 bees, respectively, were used in each experiment. The bees were collected at random from a hive. The cages containing the bees were kept at 30° C. for 1 hr. before being transferred to a cabinet at the experimental temperature, at which they remained for 3 days. Three experiments were carried out at each of a range of temperatures from 0–40° C. at intervals of 5° C.; only the mean results obtained at each temperature are given below.

On the first day of an experiment the cages were transferred to the appropriate constant temperature cabinet at 11.30 hr. and readings taken every subsequent hour until 17.30 hr. On the two following days readings were taken hourly from 09.00 to 17.30 hr. and on the final day of the experiment from 09.00 hr. to 14.30 hr. The temperatures inside the cages did not approach stability until an hour or so after they had been placed in the cabinet, so readings taken before 14.30 hr. on the first day have been ignored unless otherwise stated. Whenever possible, the data given for each experiment are those obtained during three consecutive 24 hr. periods (to be referred to as days 1, 2 and 3, respectively) starting at 14.30 hr. on the first day. Experiments at the three lowest temperatures or at the highest temperature often had to be discontinued before the 3 days had elapsed owing to the inability of the bees to survive.

No attempt was made to control humidity during the experiments.

RESULTS

Death-rate

The mean percentage of bees dead or immobile at the end of day 1 is given in Table 1. In the temperature range of 25–35° C. the percentage was low and about

Table 1. *Mean percentage of bees dead or immobile at end of day 1*

Environmental temperature (° C.)	No. of bees in a group				
	10	25	50	100	200
0	100	100	100	100	90
5	100	100	100	100	34
10	100	73	43	42	5
15	10	21	11	9	3
20	10	12	6	4	3
25	3	3	1	1	2
30	20	11	3	4	1
35	3	0	3	4	2
40	10	12	9	14	47

the same in groups of different sizes. (The relatively high death-rate in the cages containing 10 and 25 bees at 30° C. is mostly due to the large percentage that died in one experiment—50% and 16% respectively.) It was found that, below 25° C., the larger the number of bees in a cage, the greater the percentage surviving at the end of day 1. However, at 40° C. relatively fewer of the bees kept in groups of 200

survived than those kept in smaller groups, although in no instance significantly so. Only in one of the three experiments at 40° C. did more than 50% of the bees kept in groups of 200 survive day 1, and even then over 100 bees were dead by the end of day 2.

At 15–35° C. most groups survived until the end of day 3, but at 10° C. and below only few of the groups did so. The exact number of hours from the time they were first placed in the experimental cabinet, before the bees in the different groups all became dead or immobile, is not always known since this often occurred outside the hours of observation. However, at 0° C. groups of 10 or 25 bees only survived 1 or 2 hr., and sometimes only half an hour, groups of 50 or 100 bees survived about 4–5 hr., and groups of 200 bees over 6 hr. but generally less than 21½ hr. At 5° C. groups of 10 bees still survived 1 or 2 hr., groups of 25 bees about 5 hr., groups of 50 or 100 bees over 6 hr., but generally less than 24 hr., and groups of 200 bees for 2 or 3 days. At 10° C. groups of 10 bees survived about 5 hr., groups of 25 bees 1 or 2 days, and the larger groups generally survived all 3 days of an experiment.

Temperatures of the groups

The temperatures maintained by groups of different sizes at different environmental temperatures are shown in Fig. 1. Whenever possible the data presented are the mean group temperatures recorded during days 1, 2 and 3. However, as noted above, groups kept at 40° C. or at 10° C. and below often succumbed long before 3 days had elapsed. Consequently, at these environmental temperatures only the mean temperature in each cage 3 hr. after introduction to the cabinet concerned is given (i.e. the mean temperature at 14.30 hr. on day 1).

The temperatures of the groups increased with the environmental temperature. At temperatures ranging from 0–30° C. they kept themselves above the temperature of the environment for as long as they survived—in general the lower the environmental temperature the greater the difference between it and that of the groups of bees. Thus on the three occasions that a group of 200 bees was kept at 5° C. its mean temperature during days 1 and 2 was 20.5° C., i.e. 15.5° C. higher than the environment.

It is apparent that the larger the group the higher its temperature above that of the environment. The difference between the temperatures of the larger and smaller groups in each experiment decreased with increase in environmental temperature. At 35° C. or 40° C. all groups were at approximately the same temperature, i.e. at or slightly below the environmental temperature.

Percentage of bees clustering

During hourly readings the approximate percentage of bees clustering round the ends of the feeders and the bulb of the thermometer in each cage was recorded. The mean percentage of bees in the different groups that were clustering at each environmental temperature is given in Table 2. The results presented for the experiments at 40° C. are those obtained on day 1 only, and the data given for 10° C.

are from the one experiment in which the groups of 50, 100 and 200 bees survived without considerable loss until the end of day 3.

In the temperature range of 20–40° C. the percentage of bees clustering increased with increase in the size of their groups. At 15° C. and below there was a marked

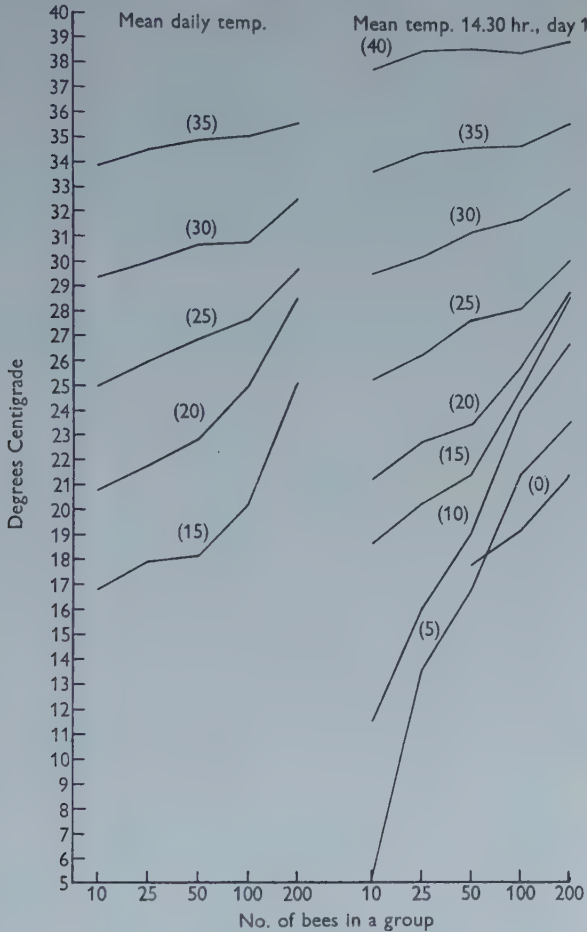


Fig. 1. Temperatures of groups at various environmental temperatures (environmental temperatures in parentheses).

Table 2. Mean percentages of bees recorded clustering

Environmental temperature (° C.)	No. of bees in a group				
	10	25	50	100	200
10	—	—	99	99	98
15	70	81	73	78	81
20	1	2	40	48	48
25	0	1	20	47	64
30	1	2	23	46	57
35	0	4	24	48	55
40	0	0	11	44	56

increase in the percentage of bees clustering in groups of all sizes, particularly in the cases of bees in groups of 10 or 25 which at higher temperatures clustered little if at all. At environmental temperatures of 10° C. and below usually well over 90% of the bees clustered before they became chilled and dropped on to the floor of their cage.

Amount of syrup and water consumed

The amounts of syrup and water consumed daily per bee in the different groups at different environmental temperatures are given in Tables 3 and 4 respectively. The data presented for experiments carried out at temperatures from 15–35° C. are the mean of those obtained on 3 days, whereas the data for experiments at 10 and 40° C. represent those obtained on 1 or 2 days only, depending on the survival time of the groups concerned.

Table 3. *Amount of sugar syrup (mm.³) consumed daily per bee*

Environmental temperature (° C.)	No. of bees in a group				
	10	25	50	100	200
10	—	39.9	50.2	50.2	50.8
15	47.5	52.4	58.4	54.6	55.7
20	39.9	45.9	38.2	37.7	37.7
25	30.0	30.6	27.8	21.3	22.4
30	32.8	23.5	13.6	11.5	17.5
35	22.9	15.8	18.0	18.6	14.2
40	3.3	1.6	6.0	5.5	11.5

Table 4. *Amount of water (mm.³) consumed per bee*

Environmental temperature (° C.)	No. of bees in a group				
	10	25	50	100	200
10	—	0.3	0.0	0.0	0.0
15	0.0	0.0	0.0	0.0	0.0
20	0.0	0.0	0.0	0.0	0.3
25	0.5	0.5	0.5	0.3	0.3
30	0.0	0.0	0.5	0.5	0.8
35	5.8	9.8	10.9	10.1	11.4
40	10.1	20.4	29.7	16.7	21.7

In each experiment the amount of syrup or water consumed in the different cages was corrected for evaporation from the feeders and change in volume according to temperature, by subtracting from the results obtained the amount lost from the feeders in a control cage without bees. The amount of syrup or water consumed daily per bee is calculated on the mean number of bees alive in the group concerned during each day.

In all groups the amount of syrup consumed decreased with increase in environmental temperature. When the environmental temperature was 15° C. there was no correlation between the size of a group and the amount of syrup consumed per bee. However, at environmental temperatures of 20–35° C. bees in groups of 10

and 25 tended to consume more syrup than bees in larger groups; this is probably related to the fact that bees in the smaller groups clustered little, if at all, at these temperatures. At 40° C. there was, in general, a marked reduction in the amount of syrup consumed.

Negligible quantities of water were drunk at 10, 15 or 20° C., and only small amounts at 25 or 30° C., but at 35 and 40° C., relatively enormous quantities were taken.

DISCUSSION AND CONCLUSIONS

Pirsch (1923) found that the temperatures of the bodies of bees that were permitted limited movement only were similar to that of the air at about 30–40° C., but at an air temperature of 5.5° C., the bees' temperatures averaged 4.7° C. higher. Himmer (1925) found that the temperature of a resting bee approached that of its surroundings, although the temperature of an active bee rose considerably higher, differences of as much as 20° C. being found at times.

Himmer (1926) reported that individual bees lose motility at 11–12° C. and become rigid at 6–7° C. The authors (unpublished) have found that the temperature at which bees enter chill coma varies between 9 and 12° C. The present results illustrate the ability of a comparatively small number of bees to raise their surrounding temperature, and consequently to increase their survival time, although to a relatively small extent in comparison with a colony of normal size. Thus Corkins (1932) found that two-thirds of the members of a colony of about 17,500 bees survived after it had been kept at a mean temperature of –15° C. for 329 hr.

Various observers have reported that bees form a cluster within the hive when the outside temperature drops to below 15° C. (e.g. Phillips & Demuth, 1914; Himmer, 1926), although bees may start to cluster when the outside temperature is 18° C. (Wilson & Milum, 1927). The present results explain these observations but show they are incomplete; with decrease in the environmental temperature from 20–15° C. the percentage of bees clustering increased markedly and practically all the bees of a group were clustering at 10° C. At 20° C. and above, however, although bees in groups of 10 or 25 showed little tendency to cluster, the proportion clustering in the larger groups increased with the size of the groups. In such circumstances, therefore, clustering is not in response to low temperature, but is the result of the mutual attraction of the bees to one another. The larger the group of bees forming a cluster, the more bees are attracted to join it. These results confirm and extend those of Lecomte (1950) and Free & Butler (1955).

Many workers (e.g. Gates, 1914; Himmer, 1926; Wilson & Milum, 1927) have concluded that there is, at times, an inverse relationship between the temperature in the centre of a broodless cluster and that of the outside air, and Himmer (1932) postulated that this resulted in the maintenance of the minimum temperature necessary for movement on the outskirts of the cluster. But this relationship appears to be a short-term one only, and although the cluster temperature remains fairly constant regardless of outside temperature, such changes as do occur are directly related to the environmental temperature (e.g. Corkins, 1930; Lavie, 1954). During

the present study the temperature within the cages increased both with the number of bees present and with the environmental temperature.

There are two ways by which bees in a winter cluster may possibly compensate for a decrease in environmental temperature. First, by reduction of heat loss, both by contraction of the cluster which, as Corkins (1930) pointed out, decreases its cooling surface and, secondly, by increased heat production, i.e. increase in the bees' metabolic rate. Although the latter method has been assumed to occur by many workers who have studied the behaviour of the winter cluster (e.g. Phillips & Demuth, 1914; Himmer, 1926), the present results provide the first real evidence that bees will respond to decreased environmental temperature by increased metabolism and heat production, and consequently by increased food consumption.

In the present experiments bees drank very little water at environmental temperatures of 30° C. and below, although their food was highly concentrated sugar syrup. Considerable quantities were drunk at 35° C. and nearly twice the amount at 40° C. There appear to be two possible explanations of this. It could have been the result of the bees consuming water which was subsequently manipulated and evaporated between the mouthparts as happens within the hive when outside temperatures reach 32° C. (Lindauer, 1954). However, above temperatures which are critical for each species, the wax layer of the epicuticle of insects becomes permeable to water and their transpiration rate increases very greatly (Wigglesworth, 1953). Probably, therefore, the high water consumption by bees when kept at 35 and 40° C. was associated with a high rate of water loss through the cuticle.

The temperatures within the cages were only slightly below that of the environment at 40° C., so that any evaporation of water that occurred was relatively ineffective in lowering the temperature surrounding the bees. In contrast, Lindauer (1954) found that at an outside temperature of 40° C. the temperature within a hive containing a colony of bees was 36° C.

The high death-rate of bees kept at 40° C. may partially have been a consequence of the temperature itself, or the temperature combined with a high relative humidity which, as Wolfenbarger (1934) and Woodrow (1935) have shown, alone adversely affects longevity by causing dysentery.

SUMMARY

1. Bees have been kept in groups whose numbers ranged from 10 to 200 bees, at temperatures ranging from 0-40° C.
2. At 40° C. bees in groups of 200 had a higher death-rate than bees in smaller groups. At temperatures of 25-35° C. the death-rate was low and about the same in all groups. Below 25° C. the more bees in a group, the longer they survived.
3. The temperatures of all groups increased with that of their environment, the larger a group, the higher its temperature. The difference between the external temperature and that of the groups decreased with increase in the former until at 35 and 40° C. groups of all sizes were at or slightly below environmental temperature.

4. At temperatures from 20–40° C. the percentage of bees in a group that were clustering was directly related to the size of their group, bees in groups of 10 or 25 hardly clustering at all. At each temperature at 15° C. or below, about the same high percentage of bees clustered in all groups.

5. The amount of food (sugar syrup) consumed per bee increased with decrease in the environmental temperature. Very little water was drunk at environmental temperatures of 25° C. or lower but, at 35° C. and above, relatively enormous quantities were taken.

6. These results have been discussed especially in relation to information on the temperature regulation and food consumption of colonies in winter.

We wish to thank Dr K. Mellanby for helpful discussions during this work and Dr C. G. Butler, Mr P. S. Milne and Dr J. Simpson for their criticism of the manuscript.

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INDEX OF SUBJECTS

A

- Absorption:**
See Sugars and Tripalmitin
- Acid production:**
 After fertilization of sea-urchin eggs. A re-examination of the lactic acid hypothesis (ROTHSCHILD) 843
- Acoustic reflexion:**
 Experiments with perch (*Perca fluviatilis* Linn.) to determine the proportion of the echo returned by the swimbladder (HARDEN JONES and PEARCE) 437
- Adaptability:**
 The, of mice to high environmental temperatures (HARRISON) 892
- Adaptation:**
 Flight activity in the blowfly *Calliphora erythrocephala* in relation to light and radiant heat, with special reference to (DIGBY) I
 — Flight activity in the blowfly, *Calliphora erythrocephala* in relation to wind speed, with special reference to (DIGBY) 776
- Adaptive behaviour:**
See Temperature
- Adaptive structures:**
 The efficiency of, in the nymph of *Rhithrogena emicolorata* (Curtis) (Ephemeroptera) (STUART) 27
- Alimentary canal:**
 The absorption of glucose from the, of the locust *Schistocerca gregaria* (Forsk.) (TREHERNE) 297
- Alkaline denaturation:**
See Oxygen dissociation
- Amino-acid composition:**
 The inorganic and, of some lamellibranch muscles (POTTS) 749
- Amino-acids:**
See Malpighian tubules
- Ammonia:**
 Conductimetric method for the estimation of small quantities of (SHAW and STADDON) 85
- Ancyclus fluviatilis** (O. F. Müller):
 Seasonal and experimental variations of the oxygen consumption of the (BERG, LUMBYE and OCKELMANN) 43
- Ant:**
See *Lasius niger* L.
- Aphididae:**
See Feeding
- Aphis fabae** Scop.:
 Effects of the ant, *Lasius niger* L., on the feeding and excretion of the bean aphid (BANKS and NIXON) 703

Apis mellifera:

Observations on the temperature regulation and food consumption of honeybees (FREE and SPENCER-BOOTH) 930

Artemia salina

- (L.):
 The survival of, in various media (CROGHAN) 213
 — The osmotic and ionic regulation of (CROGHAN) 219
 — The mechanism of osmotic regulation in: the physiology of the branchiae (CROGHAN) 234
 — The mechanism of osmotic regulation in: the physiology of the gut (CROGHAN) 243
 — Ionic fluxes in (CROGHAN) 425

B

Bimalate ions:

Chemotaxis of bracken spermatozooids: the role of (BROKAW) 192

Black snake toxin:

Effect of the, on the gastrocnemius-sciatic preparation (MOHAMED and ZAKI) 20

Blood volumes:

The, of some representative molluscs (MARTIN, HARRISON, HUSTON and STEWART) 260

Blowfly:

See *Calliphora erythrocephala*

Bombyx mori

L.
In vitro culture of embryos in the silkworm.
 I. Culture in silkworm egg extract, with special reference to some characteristics of the diapausing egg (TAKAMI) 286

Bracken spermatozooids:

- Chemotaxis of: the role of bimalate ions (BROKAW) 192
 — Chemotaxis of, implications of electrochemical orientation (BROKAW) 197

Bull:

The movement of the spermatozoa of the (GRAY) 96

C

Calliphora erythrocephala:

- Flight activity in the blowfly, in relation to light and radiant heat, with special reference to adaptation (DIGBY) I
 — Flight activity in the blowfly, in relation to wind speed, with special reference to adaptation (DIGBY) 776

Carausius morosus

Br.
 The electrical and mechanical responses of the prothoracic flexor tibialis muscle of the stick insect (WOOD) 850

Carcinus maenas

- (L.):
 — Tidal and diurnal rhythms of locomotory activity in (NAYLOR) 602

Carcinas maenas (L.):

- Further studies on ionic regulation in the muscle fibres of (SHAW) 902
- Osmoregulation in the muscle fibres of (SHAW) 920

Cerianthus (Coelenterata)

- The co-ordination of the responses of (HORRIDGE) 369

Chalcid wasps:

- See *Mormoniella vitripennis* (WALKER)

Chemotaxis:

- Of bracken spermatozooids: the role of bimalate ions (BROKAW) 192
- Of bracken spermatozooids: implications of electrochemical orientation (BROKAW) 197

Chironomus riparius Meigen:

- The relation of oxygen consumption to body size and to temperature in the larvae of (EDWARDS) 383

Cleavage:

- On 'The stationary surface ring' in heart-shaped (DAN) 400
- A quantitative description of protoplasmic movement during, in the sea-urchin egg (HIRAMOTO) 407

Climatic environments:

- The growth and development of mice in three (BIGGERS, ASHOUB, McLAREN and MICHIE) 144

Cockroach:

- See *Periplaneta americana* L.

Conductimetric method:

- For the estimation of small quantities of ammonia (SHAW and STADDON) 85

Co-ordination:

- The, of responses of *Cerianthus* (Coelenterata) 369
- See Insect movements

Crocodiles:

- Nitrogenous excretion in (KHALIL and HAGGAG) 552

D**Dehydration:**

- Comparison of, and hydration of two genera of frogs (*Heleioporus* and *Neobatrachus*) that live in areas of varying aridity (BENTLEY, LEE and MAIN) 677

Development:

- See Climatic environments

Digestion:

- See Tripalmitin

Diurnal rhythms:

- See Locomotory activity

Dividing cells:

- Surface characters of: I. Stationary surface rings (ISHIZAKA) 396

Dixippus morosus:

- Excretion by the malpighian tubules of the stick insect (orthoptera, phasmidae): amino acids, sugars and urea (RAMSAY) 871

Dragonfly nymph:

- See Insect movements

Drosophila subobscura:

- The effects of temperature and of egg-laying on the longevity of (MAYNARD SMITH) 832

Dytiscus:

- See Insect movements

E**Earthworm:**

- See Oxygen dissociation

Echinodermata:

- Studies on the myoneural physiology of. III. Spontaneous activity of the pharyngeal retractor muscle of *Cucumaria* (POPLE and EWER) 712

Echo:

- See Acoustic reflexion

Efficiency:

- The, of adaptive structures in the nymph of *Rhithrogena semicolorata* (Curtis) (Ephemeroptera) (STUART) 27

Egg:

- See *Bombyx mori* L. and Sea-urchin

Egg-laying:

- The effects of temperature and of, on the longevity of *Drosophila subobscura* (MAYNARD SMITH) 832

Electric organs:

- On the function and evolution of, in fish (LISSMAN) 156

Electrochemical orientation:

- See Chemotaxis

Electrolyte solutions:

- The effect of, on the hatching rate of the eggs of *Trichostrongylus retortaeformis* (Zeder) and its interpretation in terms of a proposed hatching mechanism of stronglyloid eggs (WILSON) 584

Embryonic mortality:

- in relation to ovulation rate in the house mouse (BOWMAN and ROBERTS) 138

Embryos:

- In vitro* culture of embryos in the silkworm, *Bombyx mori* L. I. Culture in silkworm egg extract with special reference to some characteristics of the diapausing egg (TAKAMI) 286

Environments, marine and brackish water:

- Oxygen consumption as a function of size and salinity in *Metapenaeus monoceros* Fab. from (RAO) 307

Enzymes:

- A survey of the, from the gastro-intestinal tract of *Helix pomatia* (MYERS and NORTH-COTE) 639

Erpobdella testacea (Sav.):

- Seasonal variation in the respiratory acclimatization of the leech (MANN) 314

Evolution:

- See Electric organs

Excretion:

By the malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmodidae): amino-acids, sugars and urea (RAMSAY) 871
 See *Aphis fabae* Scop.

Experimental production:

Of midgut tumours in *Periplaneta americana* L. (HARKER) 251

F**Facultative diapause:**

The induction and termination of, in the chalcid wasps *Mormoniella vitripennis* (Walker) and *Tritoneptis klugii* (Ratzeburg) (SCHNEIDERMAN and HORWITZ) 520

Feeding:

Studies on the, and nutrition of *Tuberolachnus salignus* (Gmelin) (Homoptera, Aphididae). II. The nitrogen and sugar composition of ingested phloem sap and excreted honeydew (MITTLER) 74

— Studies on the, and nutrition of *Tuberolachnus salignus* (Gmelin) (Homoptera, Aphididae). III. The nitrogen economy (MITTLER) 626

— See *Aphis fabae* Scop.

Fertilization:

See Sea-urchin

Fish:

See Gas secretion

— An apparatus for the study of the locomotion of (BAINBRIDGE and BROWN) 134

— On the function and evolution of electric organs in (LISSMANN) 156

Flagella:

Wave propagation along (MACHIN) 796

Flight:

Soaring and gliding, of the black vulture (NEWMAN) 280

Flight activity:

In the blowfly *Calliphora erythrocephala* in relation to light and radiant heat, with special reference to adaptation (DIGBY) 1

— In the blowfly, *Calliphora erythrocephala*, in relation to wind speed with special reference to adaptation (DIGBY) 776

Food consumption:

Observations on the temperature regulation and, of honeybees (*Apis mellifera*) (FREE and SPENCER-BOOTH) 930

Form perception:

Some experiments on, in the nymphs of the desert locust, *Schistocerca gregaria* Forskål (WALLACE) 765

Frogs:

See *Heleioporus*

G**Gas secretion:**

In fishes lacking rete mirabile (SUNDNES, ENNS and SCHOLANDER) 671

Gastrocnemius-sciatic:

See Black snake toxin

Gastro-intestinal tract:

See *Helix pomatia*

Gill ventilation:

The mechanism of, in three freshwater teleosts (HUGHES and SHELTON) 807

Glucose:

The absorption of, from the alimentary canal of the locust *Schistocerca gregaria* (Forsk.) (TREHERNE) 297

Growth:

The, and development of mice in three climatic environments (BIGGERS, ASHOUB, McLAREN and MICHIE) 144

Gymnarchus niloticus:

The mechanism of object location in, and similar fish (LISSMANN and MACHIN) 451

H**Haemoglobins:**

The oxygen dissociation and alkaline denaturation of, from two species of earthworm (HAUGHTON, KERKUT and MUNDAY) 360

Haemolymph:

Induced ovarian development in decapitated mosquitos by transfusion of (GILLETT) 685

Hatching:

The effect of weak electrolyte solutions on the hatching rate of the eggs of *Trichostrongylus retortaeformis* (Zeder) and its interpretation in terms of a proposed hatching mechanism of strongyloid eggs (WILSON) 584

Heleioporus:

Comparison of dehydration and hydration of two genera of frogs (*Heleioporus* and *Neobatrachus*) that live in areas of varying aridity (BENTLEY, LEE and MAIN) 677

Helix pomatia:

A survey of the enzymes from the gastro-intestinal tract of (MYERS and NORTHCOTE) 639

Honeybees:

See *Apis mellifera*

Hydration:

See Dehydration

Hydrophilus:

See Insect movements

Hydroxytryptamine:

The occurrence of 5-, in scorpion venom (ADAM and WEISS) 39

I**Induction:**

See Facultative diapause

Inorganic:

See Amino acid composition

Insect:

See Waterproofing mechanism

Insect movements:

The co-ordination of. III. Swimming in *Dytiscus*, *Hydrophilus* and a dragonfly nymph (HUGHES) 567

Ionic fluxes:

In *Artemia salina* (L.) (CROGHAN) 425

Ionic regulation:

See *Artemia salina* (L.)

Ions:

See Bimalate ions

L**Lamellibranch muscles:**

The inorganic and amino acid composition of some (POTTS) 749

Lampern:

See *Lampetra fluviatilis* L.

Lampetra fluviatilis L.:

The mechanism of marine osmoregulation in the lampern, and the causes of its breakdown during the spawning migration (MORRIS) 649

Lasius niger L.:

Effects of the ant, on the feeding and excretion of the bean aphid, *Aphis fabae* Scop. (BANKS and NIXON) 703

Leech:

See *Erpobdella testacea* (Sav.)

Limpet:

See *Ancylus fluviatilis*

Locomotion:

An apparatus for the study of the locomotion of fish (BAINBRIDGE and BROWN) 134

Locomotor activity:

Tidal and diurnal rhythms of, in *Carcinus maenas* (L.) (NAYLOR) 602

Locust:

See *Schistocerca gregaria*

Longevity:

The effects of temperature and of egg-laying on the, of *Drosophila subobscura* (MAYNARD SMITH) 832

M**Malpighian tubules:**

Excretion by the, of the stick insect, *Dixippus morosus* (orthoptera, phasmidae) amino-acids, sugars and urea (RAMSAY) 871

Marine osmoregulation:

The mechanism of, in the lampern (*Lampetra fluviatilis* L.) and the causes of its breakdown during the spawning migration (MORRIS) 649

Mechanism:

See Marine osmoregulation and Object location

Metabolism:

See Sugars

Metapenaeus monoceros Fab.:

Oxygen consumption as a function of size and salinity in, from marine and brackish-water environments (RAO) 307

Mice:

The adaptability of mice to high environmental temperatures (HARRISON) 892

— See Climatic environments

Midgut tumours:

Experimental production of, in *Periplaneta americana* L. (HARKER) 251

Migration:

See Spermatozoa

Molluscs:

See Blood volumes

Mormoniella vitripennis (Walker):

The induction and termination of facultative diapause in the chalcid wasps, and *Tritneptis klugii* (Ratzeburg) (SCHNEIDERMAN and HORWITZ) 520

Mosquitoes:

Induced ovarian development in decapitated, by transfusion of haemolymph (GILLET) 685

Mouse:

See Ovulation

Muscle fibres:

Osmoregulation in the, of *Carcinus maenas* (SHAW) 920

Muscles:

See Lamellibranch muscles

Myoneural physiology:

Studies on the, of echinodermata. III. Spontaneous activity of the pharyngeal retractor muscle of *Cucumaria* (POPLE and EWER) 712

Mysidium:

Turbidity and the polarized light orientation of the crustacean (BAINBRIDGE and WATERMAN) 487

N**Nemertean:**

See Worms

Neobatrachus:

See *Heleioporus*

Nitrogenous excretion:

In crocodiles (KHALIL and HAGGAG) 552

O**Object location:**

The mechanism of, in *Gymnarchus niloticus* and similar fish (LISSMANN and MACHIN) 451

Octopuses:

The influence of preoperational training on the performance of, following vertical lobe removal (WELLS and WELLS) 324
— The effect of vertical lobe removal on the performance of, in retention tests (WELLS and WELLS) 337

Opisthophthalmus latimanus Koch:

Temperature adaptive behaviour in the scorpion (ALEXANDER and EWER) 349

Orientation:

Turbidity and the polarized light, of the crustacean *Mysidium* (BAINBRIDGE and WATERMAN) 487

Osmoregulation:

In the muscle fibres of *Carcinus maenas* (SHAW) 920

Osmotic regulation:

See *Artemia salina* (L.)

Ovarian development:

Induced, in decapitated mosquitos by transfusion of haemolymph (GILLETT) 685

Ovulation:

Embryonic mortality in relation to, rate in the house mouse (BOWMAN and ROBERTS) 138

Oxygen:

Measurement of dissolved, in swamp waters. Further modification of the Winkler method (BEADLE) 556

Oxygen consumption:

- Seasonal and experimental variation of the, limpet *Ancylus fluviatilis* (O. F. Müller) (BERG, LUMBYE and OCKELMANN) 43
- As a function of size and salinity in *Metapenaeus monoceros* Fab. from marine and brackish water environments (RAO) 307
- The relation of, to body size and to temperature in the larvae of *Chironomus riparius* Meigen (EDWARDS) 383

Oxygen dissociation:

The, and alkaline denaturation of haemoglobins from two species of earthworm (HAUGHTON, KERKUT and MUNDAY) 360

P**Perca fluviatilis** Linn.:

Acoustic reflexion experiments with perch, to determine the proportion of the echo returned by the swimbladder (HARDEN JONES and PEARCE) 437

Perch:

See *Perca fluviatilis* Linn.

Periplaneta americana L.:

- Experimental production of midgut tumours in (HARKER) 251
- The digestion and absorption of tripalmitin in the cockroach (TREHERNE) 862

Polarized light:

Turbidity and the, orientation of the crustacean *Mysidium* (BAINBRIDGE and WATERMAN) 487

Preoperational training:

See *Octopus*

Prothoracic flexor tibialis muscle:

The electrical and mechanical responses of the, of the stick insect *Carausius morosus* Br. (WOOD) 850

Protoplasmic movement:

A quantitative description of, during cleavage in the sea-urchin egg (HIRAMOTO) 407

R**Respiratory acclimatization:**

Seasonal variation in the, of the leech, *Erpobdella testacea* (Sav.) (MANN) 314

Responses:

The electrical and mechanical, of the prothoracic flexor tibialis muscle of the stick insect, *Carausius morosus* Br. (WOOD) 850

Rete mirabile:

Gas secretion in fishes lacking (SUNDNES, ENNS and SCHOLANDER) 671

Retention tests:

See *Octopus*

Rhithrogena semicolorata:

The efficiency of adaptive structures in the nymph of (Curtis) (Ephemeroptera) (STUART) 27

Rhodnius prolixus Stal.:

The migration of spermatozoa in the female of (DAVEY) 694

Rhythm of reproduction:

The internal, in xerophilous birds under conditions of illumination and darkness (MARSHALL and SERVENTY) 666

S**Salinity:**

See Oxygen consumption

Schistocerca gregaria (Forsk.):

- The absorption and metabolism of some sugars in the locust (TREHERNE) 611
- Some experiments on form perception in the nymphs of the desert locust (WALLACE) 765

Scorpion:

See *Opisthophthalmus latimanus* Koch.

Scorpion venom:

The occurrence of 5-hydroxytryptamine in (ADAM and WEISS) 39

Seasonal variation:

See Respiratory acclimatization

Sea-urchin:

- A quantitative description of protoplasmic movement during cleavage in the, egg (HIRAMOTO) 407
- Acid production after fertilization of, eggs. A re-examination of the lactic acid hypothesis (ROTHSCHILD) 843

Shape:

Factors controlling the change of, of certain nemertean and turbellarian worms (CLARK and COWEY) 731

Silkworm:

See *Bombyx mori* L.

Size:

See Oxygen consumption

Spawning migration:

The mechanism of marine osmoregulation in the lampern (*Lampetra fluviatilis* L.) and the causes of its breakdown during the spawning migration (MORRIS) 649

Spermatozoa:

The migration of, in the female of *Rhodnius prolixus* Stal. (DAVEY) 694

— The movement of the, of the bull (GRAY) 96

Speed of swimming:

Of fish as related to size and to the frequency and amplitude of the tail beat (BAINBRIDGE) 109

Stationary surface ring:

See Surface characters

— On 'The', in heart-shaped cleavage (DAN) 400

Stick insect:

See *Carausius morosus* Br. and *Dixippus morosus*

Strongyloid eggs:

See Hatching

Sugars:

The absorption and metabolism of some, in the locust, *Schistocerca gregaria* (Forsk.) (TREHERNE) 611

See Malpighian tubules

Surface characters:

Of dividing cells. I. Stationary surface rings (ISHIZAKA) 396

Swamp waters:

Measurement of dissolved oxygen in, Further modification of the Winkler method (BEADLE) 556

Swimbladder:

Acoustic reflexion experiments with perch (*Perca fluviatilis* Linn.) to determine the proportion of the echo returned by the (HARDEN JONES and PEARCE) 437

Swimming:

See Insect movements

T

Tail beat:

The speed of swimming of fish as related to size and to the frequency and amplitude of the (BAINBRIDGE) 109

Teleosts:

The mechanism of gill ventilation in three freshwater (HUGHES and SHELTON) 807

— The effect of temperature changes on the thyroid-pituitary relationship in (FORTUNE) 824

Temperature:

Adaptive behaviour in the scorpion, *Opisthophthalmus latimanus* Koch (ALEXANDER and EWER) 349

— The effect of, on the waterproofing mechanism of an insect (BEAMENT) 494

— The effect of, changes on the thyroidpituitary relationship in teleosts (FORTUNE) 824

Temperature:

— The effects of, and of egg-laying on the longevity of *Drosophila subobscura* (MAYNARD SMITH) 832

— Observations on the, regulation and food consumption of honeybees (*Apis mellifera*) (FREE and SPENCER-BOOTH) 930

Temperatures:

See Mice

Termination:

See Facultative diapause

Thyroid-pituitary:

The effect of temperature changes on the, relationship in teleosts (FORTUNE) 824

Tidal rhythms:

See Locomotory activity

Toxin:

Effect of the black snake, on the gastrocnemius-sciatic preparation (MOHAMED and ZAKI) 20

Transfusion:

See Haemolymph

Trichostrongylus retortaeformis (Zeder)

See Hatching

Tripalmitin:

The digestion and absorption of, in the cockroach, *Periplaneta americana* L. (TREHERNE) 862

Tritneptis klugii (Ratzeburg):

See *Mormoniella vitripennisa* (Walker)

Tuberolachnus salignus (Gmelin):

Studies on the feeding and nutrition of (Homoptera, Aphididae). II. The nitrogen and sugar composition of ingested phloem sap and excreted honeydew (MITTLER) 74

— Studies on the feeding and nutrition of (Homoptera, Aphididae). III. The nitrogen economy (MITTLER) 626

Turbellarian:

See Worms

Turbidity:

And the polarized light orientation of the crustacean *Mysidium* (BAINBRIDGE and WATERMAN) 487

U

Urea:

See Malpighian tubules

V

Variations:

Seasonal and experimental, of the oxygen consumption of the limpet *Ancylus fluviatilis* (O. F. Müller) (BERG, LUMBYE and OCKELMANN) 43

Vertical lobe:

See *Octopus*

Vulture:

Soaring and gliding flight of the black (NEWMAN) 280

W**Waterproofing mechanism:**

The effect of temperature on the, of an
insect (BEAMENT) 494

Wave propagation:

Along flagella (MACHIN) 796

Wind speed:

See Flight activity

Winkler method:

See swamp waters

Worms:

Factors controlling the change of shape of
certain nemertean and turbellarian (CLARK
and COWEY) 731

X**Xerophilous birds:**

The internal rhythm of reproduction in,
under conditions of illumination and
darkness (MARSHALL and SERVENTY) 666

INDEX OF AUTHORS

A

- Adam, K. R. and Weiss, C.** The occurrence of 5-hydroxytryptamine in scorpion venom 39
Alexander, Anne J. and Ewer, D. W. Temperature adaptive behaviour in the scorpion, *Opisthophthalmus latimanus* Koch. 349
Ashoub, M. R. See BIGGERS, ASHOUB, McLAREN and MICHIE

B

- Bainbridge, Richard.** The speed of swimming of fish as related to size and to the frequency and amplitude of the tail beat 109
Bainbridge, Richard and Brown, R. H. J. An apparatus for the study of the locomotion of fish 134
Bainbridge, Richard and Waterman, Talbot H. Turbidity and the polarized light orientation of the crustacean *Mysidium* 487
Banks, C. J. and Nixon, H. L. Effects of the ant, *Lasius niger* L., on the feeding and excretion of the bean aphid, *Aphis fabae* Scop. 703
Beadle, L. C. Measurement of dissolved oxygen in swamp waters. Further modification of the Winkler method 556
Beament, J. W. L. The effect of temperature on the waterproofing mechanism of an insect 494
Bentley, P. J., Lee, A. K. and Main, A. R. Comparison of dehydration and hydration of two genera of frogs (*Heleioporus* and *Neobatrachus*) that live in areas of varying aridity 677
Berg, Kaj, Lumbye, Jørgen and Ockelmann, K. W. Seasonal and experimental variations of the oxygen consumption of the limpet *Ancylus fluviatilis* (O. F. Müller) 43
Biggers, J. D., Ashoub, M. R., McLaren, Anne and Michie, Donald. The growth and development of mice in three climatic environments 144
Bowman, J. C. and Roberts, R. C. Embryonic mortality in relation to ovulation rate in the house mouse 138
Brokaw, C. J. Chemotaxis of bracken spermatozooids: the role of bimalate ions 192
 — Chemotaxis of bracken spermatozooids: implications of electro-chemical orientation 197
Brown, R. H. J. See BAINBRIDGE and BROWN

C

- Clark, R. B. and Cowey, J. B.** Factors controlling the change of shape of certain nemertean and turbellarian worms 731
Cowey, J. B. See CLARK and COWEY

- Croghan, P. C.** The survival of *Artemia salina* (L.) in various media 213
 — The osmotic and ionic regulation of *Artemia salina* (L.) 219
 — The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the branchiae 234
 — The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the gut 243
 — Ionic fluxes in *Artemia salina* (L.) 425

D

- Dan, Katsuma.** On 'The stationary surface ring' in heart-shaped cleavage 400
Davey, K. G. The migration of spermatozoa in the female of *Rhodnius prolixus* Stal. 694
Digby, Peter S. B. Flight activity in the blowfly *Calliphora erythrocephala* in relation to light and radiant heat, with special reference to adaptation 1
 — Flight activity in the blowfly, *Calliphora erythrocephala*, in relation to wind speed, with special reference to adaptation 776

E

- Edwards, R. W.** The relation of oxygen consumption to body size and to temperature in the larvae of *Chironomus riparius* Meigen 383
Enns, T. See SUNDNES, ENNS and SCHOLANDER
Ewer, D. W. See ALEXANDER and EWER
 — See POPLE and EWER

F

- Fortune, P. Y.** The effect of temperature changes on the thyroid-pituitary relationship in teleosts 824
Free, J. B. and Spencer-Booth, Yvette. Observations on the temperature regulation and food consumption of honeybees (*Apis mellifera*) 930

G

- Gillett, J. D.** Induced ovarian development in decapitated mosquitos by transfusion of haemolymph 685
Gray, J. The movement of the spermatozoa of the bull 96

H

- Haggag, Gaber.** See KHALIL and HAGGAG
Harker, Janet E. Experimental production of midgut tumours in *Periplaneta americana* L. 251

- Harrison, F. M.** See MARTIN, HARRISON, HUSTON and STEWART
- Harrison, G. Ainsworth.** The adaptability of mice to high environmental temperatures 871
- Haughton, T. M., Kerkut, G. A. and Munday, K. A.** The oxygen dissociation and alkaline denaturation of haemoglobins from two species of earthworm 360
- Hiramoto, Y.** A quantitative description of protoplasmic movement during cleavage in the sea-urchin egg 407
- Horridge, G. A.** The co-ordination of the responses of *Cerianthus* (Coelenterata) 369
- Horwitz, Judith.** See SCHNEIDERMAN and HORWITZ
- Hughes, G. M.** The co-ordination of insect movements. III. Swimming in *Dytiscus*, *Hydrophilus*, and a dragonfly nymph 567
- Hughes, G. M. and Shelton, G.** The mechanism of gill ventilation in three freshwater teleosts 807
- Huston, M. J.** See MARTIN, HARRISON, HUSTON and STEWART
- I**
- Ishizaka, Shozo.** Surface characters of dividing cells. I. Stationary surface rings 396
- J**
- Jones, F. R. Harden and Pearce, G.** Acoustic reflexion experiments with perch (*Perca fluviatilis* Linn.) to determine the proportion of the echo returned by the swimbladder 437
- Kerkut, G. A.** See HAUGHTON, KERKUT and MUNDAY
- K**
- Khalil, Fouad and Haggag, Gaber.** Nitrogenous excretion in crocodiles 552
- L**
- Lee, A. K.** See BENTLEY, LEE and MAIN
- Lissmann, H. W.** On the function and evolution of electric organs in fish 156
- Lissman, H. W. and Machin, K. E.** The mechanism of object location in *Gymnarchus niloticus* and similar fish 451
- Lumbye, Jorgen.** See BERG, LUMBYE and OCKELMANN
- M**
- Machin, K. E.** Wave propagation along flagella 796
- See LISSMANN and MACHIN
- Main, A. R.** See BENTLEY, LEE and MAIN
- Mann, K. H.** Seasonal variation in the respiratory acclimatization of the leech *Erpobdella testacea* (Sav.) 314
- Marshall, A. J. and Serventy, D. J.** The internal rhythm of reproduction in xerophilous birds under conditions of illumination and darkness 666
- Martin, S. W., Harrison, F. M., Huston, M. J. and Stewart, D. M.** The blood volumes of some representative molluscs 260
- McLaren, Anne.** See BIGGERS, ASHOUB, McLAREN and MICHIE
- Michie, Donald.** See BIGGERS, ASHOUB, McLAREN and MICHIE
- Mittler, T. E.** Studies on the feeding and nutrition of *Tuberolachmus salignus* (Gmelin) (Homoptera, Aphididae). II. The nitrogen and sugar composition of ingested phloem sap and excreted honeydew 74
- Studies on the feeding and nutrition of *Tuberolachmus salignus* (Gmelin) (Homoptera, Aphididae). III. The nitrogen economy 626
- Mohamed, A. H. and Zaki, O.** Effect of the black snake toxin on the gastrocnemius-sciatic preparation 20
- Morris, R.** The mechanism of marine osmoregulation in the lampern (*Lampetra fluviatilis* L.) and the causes of its breakdown during the spawning migration 649
- Munday, K. A.** See HAUGHTON, KERKUT and MUNDAY
- Myers, Fay L. and Northcote, D. H.** A survey of the enzymes from the gastrointestinal tract of *Helix pomatia* 639
- N**
- Naylor, E.** Tidal and diurnal rhythms of locomotory activity in *Carcinus maenas* (L.) 602
- Newman, B. G.** Soaring and gliding flight of the black vulture 280
- Nixon, H. L.** See BANKS and NIXON
- Northcote, D. H.** See MYERS and NORTHCOTE
- O**
- Ockelmann, K. W.** See BERG, LUMBYE and OCKELMANN
- P**
- Pearce, G.** See JONES and PEARCE
- Pople, W. and Ewer, D. W.** Studies on the myoneural physiology of echinodermata. III. Spontaneous activity of the pharyngeal retractor muscle of *Cucumaria* 712
- Potts, W. T. W.** The inorganic and amino acid composition of some lamellibranch muscles 749
- R**
- Ramsay, J. A.** Excretion by the malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae): amino acids, sugars and urea 871

Rao, Kandula Pampapathi. Oxygen consumption as a function of size and salinity in *Metapeanaeus monoceros* Fab. from marine and brackish water environments 307

Roberts, R. C. See BOWMAN and ROBERTS

Rothschild, Lord. Acid production after fertilization of sea-urchin eggs. A re-examination of the lactic acid hypothesis 843

S

Schneiderman, Howard A. and Horwitz, Judith. The induction and termination of facultative diapause in the chalcid wasps, *Mormoniella vitripennis* (Walker) and *Tritoneptis klugii* (Ratzeburg) 520

Scholander, P. F. See SUNDNES, ENNS and SCHOLANDER

Serventy, D. J. See MARSHALL and SERVenty

Shaw, J. Further studies on ionic regulation in the muscle fibre of *Carcinus maenas* 902
— Osmoregulation in the muscle fibre of *Carcinus maenas* 920

Shaw, J. and Staddon, B. W. Conductimetric method for the estimation of small quantities of ammonia 85

Shelton, G. See HUGHES and SHELTON

Smith, J. Maynard. The effects of temperature and of egg-laying on the longevity of *Drosophila subobscura* 832

Spencer-Booth, Yvette. See FREE and SPENCER-BOOTH

Staddon, B. W. See SHAW and STADDON

Stewart, D. M. See MARTIN, HARRISON, HUSTON and STEWART

Stuart, A. M. The efficiency of adaptive structures in the nymph of *Rhithrogena semicolorata* (Curtis) (Ephemeroptera) 27

Sundnes, G., Enns, T. and Scholander, P. F. Gas secretion in fishes lacking rete mirabile 671

T

Takami, Takeo. *In vitro* culture of embryos in the silkworm, *Bombyx mori* L. I. Culture in silkworm extract with special reference to some characteristics of the diapausing egg 286

Treherne, J. E. The absorption of glucose from the alimentary canal of the locust, *Schistocerca gregaria* (Forsk.) 297

— The absorption and metabolism of some sugars in the locust, *Schistocerca gregaria* (Forsk.) 611

— The digestion and absorption of tripalmitin in the cockroach, *Periplaneta americana* L. 862

W

Wallace, G. K. Some experiments on form perception in the nymphs of the desert locust, *Schistocerca gregaria* Forskål 765

Waterman, Talbot H. See BAINBRIDGE and WATERMAN

Weiss, C. See ADAM and WEISS

Wells, M. J. and Wells, J. The influence of preoperational training on the performance of octopuses following vertical lobe removal 324

— The effect of vertical lobe removal on the performance of octopuses in retention tests 337

Wells, J. See WELLS and WELLS

Wilson, P. A. G. The effect of weak electrolyte solutions on the hatching rate of the eggs of *Trichostrogylus retortaeformis* (Zeder) and its interpretation in terms of a proposed hatching mechanism of stronglyloid eggs 584

Wood, D. W. The electrical and mechanical responses of the prothoracic flexor tibialis muscle of the stick insect, *Carausius morosus* Br. 850

Z

Zaki, O. See MOHAMED and ZAKI

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